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ATTENUATED NEGATIVE STRAND VIRUSES WITH ALTERED INTERFERON
ANTAGONIST ACTIVITY FOR USE AS VACCINES AND PHARMACEUTICALS

Abstract:

The present invention relates, in general, to attenuated negative-strand RNA viruses having an impaired ability to antagonize the cellular interferon (IFN) response, and the use of such attenuated viruses in vaccine and pharmaceutical formulations. The invention also relates to the development and use of IFN-deficient systems for selection of such attenuated viruses. In particular, the invention relates to attenuated influenza viruses having modifications to the NS1 gene that diminish or eliminate the ability of the NS1 gene product to antagonize the cellular IFN response. The mutant viruses replicate in vivo but demonstrate reduced pathogenicity, and therefore are well suited for live virus vaccines, and pharmaceutical formulations.

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(21) International Application Number: PCT/US99/13142 (22) International Filing Date: 11 June 1999 (11.06.99) (30) Priority Data: <table border="0"><tr><td>60/089,103</td><td>12 June 1998 (12.06.98)</td><td>US</td></tr><tr><td>60/108,832</td><td>18 November 1998 (18.11.98)</td><td>US</td></tr><tr><td>60/117,683</td><td>29 January 1999 (29.01.99)</td><td>US</td></tr></table> (71) Applicant: MOUNT SINAI SCHOOL OF MEDICINE OF THE CITY UNIVERSITY OF NEW YORK [US/US]; One Gustave Levy Place, New York, NY 10029-6574 (US). (72) Inventors: PALESE, Peter; 414 Highwood Avenue, Leonia, NJ 07605 (US). GARCIA-SASTRE, Adolfo; Apartment 3G, 16 East 96th Street, New York, NY 10128 (US). O'NEIL, Robert; One Gustave Levy Place, New York, NY 10029-6574 (US). (74) Agents: CORUZZI, Laura, A. et al.; Pennie & Edmonds LLP, 1155 Avenue of the Americas, New York, NY 10036 (US).		60/089,103	12 June 1998 (12.06.98)	US	60/108,832	18 November 1998 (18.11.98)	US	60/117,683	29 January 1999 (29.01.99)	US	(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
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(54) Title: NOVEL METHODS AND INTERFERON DEFICIENT SUBSTRATES FOR THE PROPAGATION OF VIRUSES											
(57) Abstract <p>The present invention relates to novel methods and substrates for the propagation of viruses. The invention relates to IFN-deficient substrates and methods for propagating viruses in these unconventional substrates. In particular, the invention relates to methods of propagating viruses in immature embryonated eggs, preferably six- to nine-day-old chicken eggs. The methods of the invention are particularly attractive for growing viruses suitable for use in vaccine and pharmaceutical formulations.</p>											

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**NOVEL METHODS AND INTERFERON
DEFICIENT SUBSTRATES FOR THE
PROPAGATION OF VIRUSES**

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5 part, by a grant from the National Institutes of Health, and
the Government may have certain rights to the invention.

This application is a continuation-in-part of
Application Serial No. 60/117,683 filed January 29, 1999;
10 Application Serial No. 60/108,832 filed November 18, 1998;
and Application Serial No. 60/089,103 filed June 12, 1998,
each of which is incorporated by reference in its entirety
herein.

15

1. INTRODUCTION

The present invention relates, in general, to attenuated
negative-strand RNA viruses having an impaired ability to
antagonize the cellular interferon (IFN) response, and the
use of such attenuated viruses in vaccine and pharmaceutical
20 formulations. The invention also relates to the development
and use of IFN-deficient systems for the selection,
identification and propagation of such attenuated viruses.

In a particular embodiment, the invention relates to
attenuated influenza viruses having modifications to the NS1
25 gene that diminish or eliminate the ability of the NS1 gene
product to antagonize the cellular IFN response. The mutant
viruses replicate in vivo, but demonstrate reduced
pathogenicity, and therefore are well suited for use in live
virus vaccines, and pharmaceutical formulations.

30

2. BACKGROUND OF THE INVENTION

2.1 THE INFLUENZA VIRUS

Virus families containing enveloped single-stranded RNA
of the negative-sense genome are classified into groups
35 having non-segmented genomes (Paramyxoviridae, Rhabdoviridae,
Filoviridae and Borna Disease Virus) or those having
segmented genomes (Orthomyxoviridae, Bunyaviridae and
Arenaviridae). The Orthomyxoviridae family, described in
detail below, and used in the examples herein, includes the

viruses of influenza, types A, B and C viruses, as well as Thogoto and Dhori viruses and infectious salmon anemia virus.

The influenza virions consist of an internal ribonucleoprotein core (a helical nucleocapsid) containing the single-stranded RNA genome, and an outer lipoprotein envelope lined inside by a matrix protein (M1). The segmented genome of influenza A virus consists of eight molecules (seven for influenza C) of linear, negative polarity, single-stranded RNAs which encode ten polypeptides, including: the RNA-dependent RNA polymerase proteins (PB2, PB1 and PA) and nucleoprotein (NP) which form the nucleocapsid; the matrix membrane proteins (M1, M2); two surface glycoproteins which project from the lipid containing envelope: hemagglutinin (HA) and neuraminidase (NA); the nonstructural protein (NS1) and nuclear export protein (NEP). Transcription and replication of the genome takes place in the nucleus and assembly occurs via budding on the plasma membrane. The viruses can reassort genes during mixed infections.

Influenza virus adsorbs via HA to sialyloligosaccharides in cell membrane glycoproteins and glycolipids. Following endocytosis of the virion, a conformational change in the HA molecule occurs within the cellular endosome which facilitates membrane fusion, thus triggering uncoating. The nucleocapsid migrates to the nucleus where viral mRNA is transcribed. Viral mRNA is transcribed by a unique mechanism in which viral endonuclease cleaves the capped 5'-terminus from cellular heterologous mRNAs which then serve as primers for transcription of viral RNA templates by the viral transcriptase. Transcripts terminate at sites 15 to 22 bases from the ends of their templates, where oligo(U) sequences act as signals for the addition of poly(A) tracts. Of the eight viral RNA molecules so produced, six are monocistronic messages that are translated directly into the proteins representing HA, NA, NP and the viral polymerase proteins, PB2, PB1 and PA. The other two transcripts undergo splicing, each yielding two mRNAs which are translated in different reading frames to produce M1, M2, NS1 and NEP. In other words, the eight viral RNA segments code for ten proteins: nine structural and one nonstructural. A summary of the

genes of the influenza virus and their protein products is shown in Table I below.

TABLE I

5

**INFLUENZA VIRUS GENOME RNA SEGMENTS AND CODING
ASSIGNMENTS^a**

	Segment	Length _b (Nucleotides)	Encoded Polypeptide _c	Length _d (Amino Acids)	Molecules Per Virion	Comments
10	1	2341	PB2	759	30-60	RNA transcriptase component; host cell RNA cap binding
	2	2341	PB1	757	30-60	RNA transcriptase component; initiation of transcription
	3	2233	PA	716	30-60	RNA transcriptase component
15	4	1778	HA	566	500	Hemagglutinin; trimer; envelope glycoprotein; mediates attachment to cells
	5	1565	NP	498	1000	Nucleoprotein; associated with RNA; structural component of RNA transcriptase
20	6	1413	NA	454	100	Neuraminidase; tetramer; envelope glycoprotein
	7	1027	M ₁	252	3000	Matrix protein; lines inside of envelope
			M ₂	96	?	Structural protein in plasma membrane; spliced mRNA
25						
	8	890	NS ₁	230		Nonstructural protein; function unknown
			NEP	121	?	Nuclear export protein; spliced mRNA
30						

^a Adapted from R.A. Lamb and P. W. Choppin (1983), Annual Review of Biochemistry, Volume 52, 467-506.

^b For A/PR/8/34 strain

35 ^c Determined by biochemical and genetic approaches

^d Determined by nucleotide sequence analysis and protein sequencing

The influenza A virus genome contains eight segments of single-stranded RNA of negative polarity, coding for one

nonstructural and nine structural proteins. The nonstructural protein NS1 is abundant in influenza virus infected cells, but has not been detected in virions. NS1 is a phosphoprotein found in the nucleus early during infection and also in the cytoplasm at later times of the viral cycle (King et al., 1975, Virology 64: 378). Studies with temperature-sensitive (ts) influenza mutants carrying lesions in the NS gene suggested that the NS1 protein is a transcriptional and post-transcriptional regulator of mechanisms by which the virus is able to inhibit host cell gene expression and to stimulate viral protein synthesis. Like many other proteins that regulate post-transcriptional processes, the NS1 protein interacts with specific RNA sequences and structures. The NS1 protein has been reported to bind to different RNA species including: vRNA, poly-A, U6 snRNA, 5' untranslated region as of viral mRNAs and ds RNA (Qiu et al., 1995, RNA 1: 304; Qiu et al., 1994, J. Virol. 68: 2425; Hatada Fukuda 1992, J Gen Virol. 73:3325-9. Expression of the NS1 protein from cDNA in transfected cells has been associated with several effects: inhibition of nucleo-cytoplasmic transport of mRNA, inhibition of pre-mRNA splicing, inhibition of host mRNA polyadenylation and stimulation of translation of viral mRNA (Fortes, et al., 1994, EMBO J. 13: 704; Enami, et al, 1994, J. Virol. 68: 1432; de la Luna, et al., 1995, J. Virol. 69:2427; Lu, et al., 1994, Genes Dev. 8:1817; Park, et al., 1995, J. Biol Chem. 270, 28433; Nemeroff et al., 1998, Mol. Cell. 1:991; Chen, et al., 1994 EMBO J. 18:2273-83).

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2.2 ATTENUATED VIRUSES

Inactivated virus vaccines are prepared by "killing" the viral pathogen, e.g., by heat or formalin treatment, so that it is not capable of replication. Inactivated vaccines have limited utility because they do not provide long lasting immunity and, therefore, afford limited protection. An alternative approach for producing virus vaccines involves the use of attenuated live virus vaccines. Attenuated viruses are capable of replication but are not pathogenic, and, therefore, provide for longer lasting immunity and afford greater protection. However, the conventional methods

for producing attenuated viruses involve the chance isolation of host range mutants, many of which are temperature sensitive; e.g., the virus is passaged through unnatural hosts, and progeny viruses which are immunogenic, yet not pathogenic, are selected.

A conventional substrate for isolating and growing influenza viruses for vaccine purposes are embryonated chicken eggs. Influenza viruses are typically grown during 2-4 days at 37°C in 10-11 day old eggs. Although most of the human primary isolates of influenza A and B viruses grow better in the amniotic sac of the embryos, after 2 to 3 passages the viruses become adapted to grow in the cells of the allantoic cavity, which is accessible from the outside of the egg (Murphy, B.R., and R.G. Webster, 1996.

Orthomyxoviruses p. 1397-1445. In Fields Virology. Lippincott-Raven P.A.)

Recombinant DNA technology and genetic engineering techniques, in theory, would afford a superior approach to producing an attenuated virus since specific mutations could be deliberately engineered into the viral genome. However, the genetic alterations required for attenuation of viruses are not known or predictable. In general, the attempts to use recombinant DNA technology to engineer viral vaccines have mostly been directed to the production of subunit vaccines which contain only the protein subunits of the pathogen involved in the immune response, expressed in recombinant viral vectors such as vaccinia virus or baculovirus. More recently, recombinant DNA techniques have been utilized in an attempt to produce herpes virus deletion mutants or polioviruses which mimic attenuated viruses found in nature or known host range mutants. Until 1990, the negative strand RNA viruses were not amenable to site-specific manipulation at all, and thus could not be genetically engineered.

Attenuated live influenza viruses produced thus far might not be capable of suppressing the interferon response in the host in which they replicate. Therefore, although these viruses are beneficial because they are immunogenic and not pathogenic, they are difficult to propagate in conventional substrates for the purposes of making vaccines.

Furthermore, attenuated viruses may possess virulence characteristics that are so mild as to not allow the host to mount an immune response sufficient to meet subsequent challenges.

5

3. SUMMARY OF THE INVENTION

The present invention relates to attenuated negative strand RNA viruses having an impaired ability to antagonize the cellular IFN response, and the use of such viruses in vaccine and pharmaceutical formulations. The mutant viruses with an impaired IFN antagonist activity are attenuated -- they are infectious, can replicate in vivo to provide subclinical levels of infection, and are not pathogenic. Therefore, they are ideal candidates for live virus vaccines. Moreover, the attenuated viruses can induce a robust IFN response which has other biological consequences in vivo, affording protection against subsequent infectious diseases and/or inducing antitumor responses. Therefore, the attenuated viruses can be used pharmaceutically, for the prevention or treatment of other infectious diseases, cancer in high risk individuals, and/or IFN-treatable diseases.

The negative strand RNA viruses used in accordance with the invention include both segmented and non-segmented viruses; preferred embodiments include but are not limited to influenza virus, respiratory syncytial virus (RSV), Newcastle disease virus (NDV), vesicular stomatitis virus (VSV), and parainfluenza virus (PIV). The viruses used in the invention may be selected from naturally occurring strains, variants or mutants; mutagenized viruses (e.g., generated by exposure to mutagens, repeated passages and/or passage in non-permissive hosts); reassortants (in the case of segmented viral genomes); and/or genetically engineered viruses (e.g. using the "reverse genetics" techniques) having the desired phenotype -- i.e., an impaired ability to antagonize the cellular IFN response. The mutant or genetically engineered virus can be selected based on differential growth in IFN deficient systems versus IFN competent systems. For example, viruses which grow in an IFN deficient system, but not in an

IFN competent system (or which grow less well in an IFN competent system) can be selected.

The attenuated virus so selected can itself be used as the active ingredient in vaccine or pharmaceutical formulations. Alternatively, the attenuated virus can be used as the vector or "backbone" of recombinantly produced vaccines. To this end, the "reverse genetics" technique can be used to engineer mutations or introduce foreign epitopes into the attenuated virus, which would serve as the "parental" strain. In this way, vaccines can be designed for immunization against strain variants, or in the alternative, against completely different infectious agents or disease antigens. For example, the attenuated virus can be engineered to express neutralizing epitopes of other preselected strains. Alternatively, epitopes of viruses other than negative strand RNA viruses can be built into the attenuated mutant virus (e.g., gp160, gp120, or gp41 of HIV). Alternatively, epitopes of non-viral infectious pathogens (e.g., parasites, bacteria, fungi) can be engineered into the virus. In yet another alternative, cancer vaccines can be prepared, e.g. by engineering tumor antigens into the attenuated viral backbone.

In a particular embodiment involving RNA viruses with segmented genomes, reassortment techniques can be used to transfer the attenuated phenotype from a parental segmented RNA virus strain (a natural mutant, a mutagenized virus, or a genetically engineered virus) to a different virus strain (a wild-type virus, a natural mutant, a mutagenized virus, or a genetically engineered virus).

The attenuated viruses, which induce robust IFN responses in hosts, may also be used in pharmaceutical formulations for the prophylaxis or treatment of other viral infections, or IFN-treatable diseases, such as cancer. In this regard, the tropism of the attenuated virus can be altered to target the virus to a desired target organ, tissue or cells in vivo or ex vivo. Using this approach, the IFN response can be induced locally, at the target site, thus avoiding or minimizing the side effects of systemic IFN treatments. To this end, the attenuated virus can be

engineered to express a ligand specific for a receptor of the target organ, tissue or cells.

The invention is based, in part, on the Applicants' discovery that NS1 of wild type influenza virus functions as
5 an IFN antagonist, in that NS1 inhibits the IFN mediated response of virus-infected host cells. Viral mutants deficient for NS1 activity were found to be potent inducers of the cellular IFN response, and demonstrated an attenuated phenotype in vivo; i.e. the mutant viruses replicate in vivo,
10 but have reduced pathogenic effects. While not intending to be bound to any theory or explanation for how the invention works, the attenuated features of the viruses of the invention are presumably due to their ability to induce a robust cellular IFN response, and their impaired ability to
15 antagonize the host IFN response. However, the beneficial features of the attenuated viruses of the invention may not be solely attributable to the effects on the cellular interferon response. Indeed, alterations in other activities associated with NS1 may contribute to the desired attenuated
20 phenotype.

The mutant influenza viruses with impaired IFN antagonist activity were shown to replicate in vivo generating titers that are sufficient to induce immunological and cytokine responses. For example, vaccination with
25 attenuated influenza virus reduced viral titer in animals that were subsequently challenged with wild-type influenza virus. The attenuated influenza viruses also demonstrated antiviral and antitumor activity. Pre-infection with attenuated influenza virus inhibited replication of other
30 strains of wild type influenza virus, and other viruses (such as Sendai virus) superinfected in embryonated eggs. Inoculation of the attenuated influenza in animals injected with tumor cells reduced the number of foci formed. Because influenza virus is known to induce a CTL (cytotoxic T
35 lymphocyte) response, the attenuated virus is a very attractive candidate for cancer vaccines.

Mutations which diminish but do not abolish the IFN antagonist activity of the virus are preferred for vaccine formulations -- such viruses can be selected for growth in both conventional and non-conventional substrates, and for

intermediate virulence. In particular, the Applicants have demonstrated that an NS1 C-terminal-truncation mutant replicates to high titers in IFN deficient substrates, such as 6 and 7-day-old embryonated chicken eggs, as well as in the allantoic membrane of 10-day-old embryonated chicken eggs, the conventional substrate for influenza virus that does not permit the growth of influenza virus mutants in which the entire NS1 gene is deleted (also referred to herein as "knockout" mutants). However, replication of the NS1-C terminal truncation mutant is diminished in 12-day-old embryonated eggs. This approach allows, for the first time, the generation and identification of live attenuated negative strand RNA viruses that have altered, but not abolished, IFN antagonist activity, and that are able to grow in substrates suitable for vaccine preparation. This approach also allows for the first time, an efficient selection identification system for influenza or other viruses which contain mutations that confer altered, but not abolished, interferon antagonist activity.

The invention also relates to the use of IFN deficient systems to propagate the attenuated viruses that cannot be grown in conventional systems currently used for vaccine production. The term "IFN-deficient systems" as used herein refers to systems, e.g., cells, cell lines and animals, such as mice, chickens, turkeys, rabbits, rats, etc., which do not produce IFN or produce low levels of IFN, do not respond or respond less efficiently to IFN, and/or are deficient in the activity of antiviral genes induced by IFN. To this end, Applicants have identified or designed a number of IFN-deficient systems that can be used, including but not limited to young embryonated eggs, IFN-deficient cell lines (such as VERO cells or genetically engineered cell lines such as STAT1 knockouts). Alternatively, embryonated eggs or cell lines can be pretreated with compounds that inhibit the IFN system (including drugs, antibodies, antisense, ribozymes, etc.). Yet another embodiment involves the use of eggs deficient in the IFN system, e.g., eggs produced by STAT1 negative birds, especially fowl, including but not limited to transgenic chickens, ducks or turkeys.

4. DESCRIPTION OF THE FIGURES

Fig. 1. DelNS1 virus inhibits wild-type influenza A virus replication in eggs. Ten-day-old embryonated chicken eggs were inoculated with the indicated pfu of delNS1 virus. Eight hours later, the eggs were infected with 10^3 pfu of WSN virus. After two days of incubation at 37°C , the allantoic fluid was harvested and WSN virus titers were determined by plaque assay in MDBK cells. Results are the average of two eggs.

Fig. 2. Induction of an antiviral response in embryonated eggs by delNS1 virus. Ten-day-old embryonated chicken eggs were inoculated with PBS (untreated) or with 2×10^4 pfu of delNS1 virus (delNS1 treated). Eight hours later, the eggs were now infected with 10^3 pfu of influenza A/WSN/33 (H1N1) virus, influenza A/PR8/34 (H+N1) virus, influenza A/X-31 (H3N2) virus, influenza B/Lee/40 virus, or Sendai virus. After two days of incubation, the allantoic fluid was harvested and virus titers were determined by a hemagglutination assay. Results are the average of two eggs.

Fig. 3. CV1 cells were transfected with a plasmid expressing IRF-3 fused to the green fluorescent protein (GFP). This allowed determining the localization of IRF-3 inside the cells by fluorescence microscopy. In some cases, an NS1 expression plasmid was cotransfected with the IRF-3 expression plasmid at the indicated ratios. 24 hours posttransfection cells were infected at high moi with PR8(WT) or with delNS1 virus as indicated. 10 hours postinfection, cells were analyzed in a fluorescence microscope for IRF-3-GFP localization. The percentage of cells showing exclusive cytoplasmic localization (CYT) and both cytoplasmic and nuclear localizations of IRF-3 (Nuc+Cyt) are indicated.

5. DETAILED DESCRIPTION OF THE INVENTION

The invention relates to the generation, selection and identification of attenuated negative strand RNA viruses that have an impaired ability to antagonize the cellular IFN response, and the use of such viruses in vaccine and pharmaceutical formulations.

The viruses can have segmented or non-segmented genomes and can be selected from naturally occurring strains, variants or mutants; mutagenized viruses (e.g., by exposure to UV irradiation, mutagens, and/or passaging); reassortants
5 (for viruses with segmented genomes); and/or genetically engineered viruses. For example, the mutant viruses can be generated by natural variation, exposure to UV irradiation, exposure to chemical mutagens, by passaging in non-permissive hosts, by reassortment (i.e., by coinfection of an
10 attenuated segmented virus with another strain having the desired antigens), and/or by genetic engineering (e.g., using "reverse genetics"). The viruses selected for use in the invention have defective IFN antagonist activity and are attenuated; i.e., they are infectious and can replicate in
15 vivo, but only generate low titers resulting in subclinical levels of infection that are non-pathogenic. Such attenuated viruses are ideal candidates for live vaccines.

In a preferred embodiment, the attenuated viruses selected for use in the invention should be capable of
20 inducing a robust IFN response in the host -- a feature which contributes to the generation of a strong immune response when used as a vaccine, and which has other biological consequences that make the viruses useful as pharmaceutical agents for the prevention and/or treatment of other viral
25 infections, or tumor formation in high risk individuals, or other diseases which are treated with IFN.

The invention is based, in part, on a number of discoveries and observations made by the Applicants when working with influenza virus mutants. However, the
30 principles can be analogously applied and extrapolated to other segmented and non-segmented negative strand RNA viruses including, but not limited to paramyxoviruses (Sendai virus, parainfluenza virus, mumps, Newcastle disease virus), morbillivirus (measles virus, canine distemper virus and
35 rinderpest virus); pneumovirus (respiratory syncytial virus and bovine respiratory virus); and rhabdovirus (vesicular stomatitis virus and lyssavirus).

First, the IFN response is important for containing viral infection in vivo. The Applicants found that growth of wild-type influenza virus A/WSN/33 in IFN-deficient mice

(STAT1-/- mice) resulted in pan-organ infection; i.e., viral infection was not confined to the lungs as it is in wild-type mice which generate an IFN response (Garcia-Sastre, et al., 1998, J. Virol. 72:8550, which is incorporated by reference
5 herein in its entirety). Second, the Applicants established that NS1 of influenza virus functions as an IFN antagonist. The Applicants discovered that an influenza virus mutant deleted of the entire NS1 gene (i.e., an NS1 "knockout") was not able to grow to high titers in IFN-competent host cells,
10 and could only be propagated in IFN-deficient hosts. The NS1 knockout virus demonstrated an attenuated phenotype (i.e., it was lethal in IFN deficient STAT1-/- mice, but not in wild-type mice) and was found to be a potent inducer of IFN responses in host cells. (Garcia-Sastre, et al., 1998,
15 Virology 252:324-330, which is incorporated by reference herein in its entirety). Preinfection with the NS1 knockout mutant virus reduced titers of wild-type influenza and other viruses (e.g., Sendai) superinfected in embryonated eggs. In another experiment, infection with the NS1 knockout mutant
20 influenza virus reduced foci formation in animals inoculated with tumor cells. Thus, the NS1 knockout influenza virus demonstrated interesting biological properties. However, the NS1 knockout mutant viruses could not be propagated in conventional systems for vaccine production. To overcome
25 this problem, the Applicants used and developed IFN-deficient systems that allow for production of reasonable yields of attenuated virus.

In addition, the Applicants designed deletion mutants of NS1, which do not delete the entire gene. Surprisingly,
30 these NS1 mutants were found to display an "intermediate" phenotype -- the virus can be grown in conventional hosts used for propagating influenza virus (although growth is better in the IFN-deficient systems which yield higher titers). Most importantly, the deletion mutants are
35 attenuated in vivo, and induce a robust IFN response. Vaccination with the influenza virus NS1 truncated mutants resulted in low titers of virus in animals subsequently challenged with wild-type virus, and afforded protection against disease.

The present invention also relates to the substrates designed for the isolation, identification and growth of viruses for vaccine purposes. In particular, interferon-deficient substrates for efficiently growing influenza virus mutants are described. In accordance with the present invention, an interferon-deficient substrate is one that is defective in its ability to produce or respond to interferon. The substrate of the present invention may be used for the growth of any number of viruses which may require interferon-deficient growth environment. Such viruses may include, but are not limited to paramyxoviruses (Sendai virus, parainfluenza virus, mumps, Newcastle disease virus), morbillivirus (measles virus, canine distemper virus and rinderpest virus); pneumovirus (respiratory syncytial virus and bovine respiratory virus); rhabdovirus (vesicular stomatitis virus and lyssavirus).

The invention also relates to the use of the attenuated virus of the invention in vaccines and pharmaceutical preparations for humans or animals. In particular, the attenuated viruses can be used as vaccines against a broad range of viruses and/or antigens, including but not limited to antigens of strain variants, different viruses or other infectious pathogens (e.g., bacteria, parasites, fungi), or tumor specific antigens. In another embodiment, the attenuated viruses, which inhibit viral replication and tumor formation, can be used for the prophylaxis or treatment of infection (viral or nonviral pathogens) or tumor formation or treatment of diseases for which IFN is of therapeutic benefit. Many methods may be used to introduce the live attenuated virus formulations to a human or animal subject to induce an immune or appropriate cytokine response. These include, but are not limited to, intranasal, intratracheal, oral, intradermal, intramuscular, intraperitoneal, intravenous and subcutaneous routes. In a preferred embodiment, the attenuated viruses of the present invention are formulated for delivery intranasally.

5.1 GENERATION OF MUTANTS WITH ALTERED IFN ANTAGONIST ACTIVITY

Any mutant virus or strain which has a decreased IFN antagonist activity can be selected and used in accordance with the invention. In one embodiment, naturally occurring mutants or variants, or spontaneous mutants can be selected that have an impaired ability to antagonize the cellular IFN response. In another embodiment, mutant viruses can be generated by exposing the virus to mutagens, such as ultraviolet irradiation or chemical mutagens, or by multiple passages and/or passage in non-permissive hosts. Screening in a differential growth system can be used to select for those mutants having impaired IFN antagonist function. For viruses with segmented genomes, the attenuated phenotype can be transferred to another strain having a desired antigen by reassortment, (*i.e.*, by coinfection of the attenuated virus and the desired strain, and selection for reassortants displaying both phenotypes).

In another embodiment, mutations can be engineered into a negative strand RNA virus such as influenza, RSV, NDV, VSV and PIV, using "reverse genetics" approaches. In this way, natural or other mutations which confer the attenuated phenotype can be engineered into vaccine strains. For example, deletions, insertions or substitutions of the coding region of the gene responsible for IFN antagonist activity (such as the NS1 of influenza) can be engineered. Deletions, substitutions or insertions in the non-coding region of the gene responsible for IFN antagonist activity are also contemplated. To this end, mutations in the signals responsible for the transcription, replication, polyadenylation and/or packaging of the gene responsible or the IFN-antagonist activity can be engineered. For example, in influenza, such modifications can include but are not limited to: substitution of the non-coding regions of an influenza A virus gene by the non-coding regions of an influenza B virus gene (Muster, et al., 1991, Proc. Natl. Acad. Sci. USA, 88:5177), base pairs exchanges in the non-coding regions of an influenza virus gene (Fodor, et al., 1998, J Virol. 72:6283), mutations in the promoter region of an influenza virus gene (Piccone, et al., 1993, Virus Res.

28:99; Li, et al., 1992, J Virol. 66:4331), substitutions and deletions in the stretch of uridine residues at the 5' end of an influenza virus gene affecting polyadenylation (Luo, et al., 1991, J Virol. 65:2861; Li, et al., J Virol. 1994, 5 68:1245). Such mutations, for example to the promoter, could down-regulate the expression of the gene responsible for IFN antagonist activity. Mutations in viral genes which may regulate the expression of the gene responsible for IFN antagonist activity are also within the scope of viruses that 10 can be used in accordance with the invention.

The present invention also relates to mutations to the NS1 gene segment that may not result in an altered IFN antagonist activity or an IFN-inducing phenotype but rather results in altered viral functions and an attenuated 15 phenotype e.g., altered inhibition of nuclear export of poly(A)-containing mRNA, altered inhibition of pre-mRNA splicing, altered inhibition of the activation of PKR by sequestering of dsRNA, altered effect on translation of viral RNA and altered inhibition of polyadenylation of host mRNA 20 (e.g., see Krug in Textbook of Influenza, Nicholson et al. Ed. 1998, 82-92, and references cited therein).

The reverse genetics technique involves the preparation of synthetic recombinant viral RNAs that contain the non-coding regions of the negative strand virus RNA which are 25 essential for the recognition by viral polymerases and for packaging signals necessary to generate a mature virion. The recombinant RNAs are synthesized from a recombinant DNA template and reconstituted in vitro with purified viral polymerase complex to form recombinant ribonucleoproteins 30 (RNPs) which can be used to transfect cells. A more efficient transfection is achieved if the viral polymerase proteins are present during transcription of the synthetic RNAs either in vitro or in vivo. The synthetic recombinant RNPs can be rescued into infectious virus particles. The 35 foregoing techniques are described in U.S. Patent No. 5,166,057 issued November 24, 1992; in U.S. Patent No. 5,854,037 issued December 29, 1998; in European Patent Publication EP 0702085A1, published February 20, 1996; in U.S. Patent Application Serial No. 09/152,845; in International Patent Publications PCT WO97/12032 published

April 3, 1997; WO96/34625 published November 7, 1996; in European Patent Publication EP-A780475; WO 99/02657 published January 21, 1999; WO 98/53078 published November 26, 1998; WO 98/02530 published January 22, 1998; WO 99/15672 published April 1, 1999; WO 98/13501 published April 2, 1998; WO 97/06270 published February 20, 1997; and EPO 780 475A1 published June 25, 1997, each of which is incorporated by reference herein in its entirety.

Attenuated viruses generated by the reverse genetics approach can be used in the vaccine and pharmaceutical formulations described herein. Reverse genetics techniques can also be used to engineer additional mutations to other viral genes important for vaccine production -- i.e., the epitopes of useful vaccine strain variants can be engineered into the attenuated virus. Alternatively, completely foreign epitopes, including antigens derived from other viral or non-viral pathogens can be engineered into the attenuated strain. For example, antigens of non-related viruses such as HIV (gp160, gp120, gp41) parasite antigens (e.g., malaria), bacterial or fungal antigens or tumor antigens can be engineered into the attenuated strain. Alternatively, epitopes which alter the tropism of the virus in vivo can be engineered into the chimeric attenuated viruses of the invention.

In an alternate embodiment, a combination of reverse genetics techniques and reassortant techniques can be used to engineer attenuated viruses having the desired epitopes in segmented RNA viruses. For example, an attenuated virus (generated by natural selection, mutagenesis or by reverse genetics techniques) and a strain carrying the desired vaccine epitope (generated by natural selection, mutagenesis or by reverse genetics techniques) can be co-infected in hosts that permit reassortment of the segmented genomes. Reassortants that display both the attenuated phenotype and the desired epitope can then be selected.

In another embodiment, the virus to be mutated is a DNA virus (e.g., vaccinia, adenovirus, baculovirus) or a positive strand RNA virus (e.g., polio virus). In such cases, recombinant DNA techniques which are well known in the art may be used (e.g., see U.S. Patent No. 4,769,330 to Paoletti,

U.S. Patent No. 4,215,051 to Smith each of which is incorporated herein by reference in its entirety).

Any virus may be engineered in accordance with the present invention, including but not limited to the families set forth in Table 2 below.

TABLE 2
FAMILIES OF HUMAN AND ANIMAL VIRUSES

<u>VIRUS CHARACTERISTICS</u>		<u>VIRUS FAMILY</u>
10	dsDNA	
	Enveloped	Poxviridae Iridoviridae Herpesviridae
	Nonenveloped	Adenoviridae Papovaviridae Hepadnaviridae
15	ssDNA	
	Nonenveloped	Parvoviridae
	dsRNA	
	Nonenveloped	Reoviridae Birnaviridae
	ssRNA	
	Enveloped	
20	Positive-Sense Genome	
	No DNA Step in Replication	Togaviridae Flaviviridae Coronaviridae Hepatitis C Virus
	DNA Step in Replication	Retroviridae
25	Negative-Sense Genome	
	Non-Segmented Genome	Paramyxoviridae Rhabdoviridae Filoviridae
	Segmented Genome	Orthomyxoviridae Bunyaviridae Arenaviridae
	Nonenveloped	Picornaviridae Caliciviridae
30		

Abbreviations used: ds = double stranded; ss = single stranded; enveloped = possessing an outer lipid bilayer derived from the host cell membrane; positive-sense genome = for RNA viruses, genomes that are composed of nucleotide sequences that are directly translated on ribosomes, = for DNA viruses, genomes that are composed of nucleotide sequences that are the same as the mRNA; negative-sense genome = genomes that are composed of nucleotide sequences complementary to the positive-sense strand.

In a preferred embodiment, the present invention relates to genetically engineered influenza viruses containing deletions and/or truncations of the NS1 gene product. NS1 mutants of influenza A and B are particularly preferred. In

one approach, portions of the amino terminal region of the NS1 gene product are retained whereas portions of the C-terminal region of the NS1 gene product are deleted. Specific desired mutations can be engineered by way of
5 nucleic acid insertion, deletion, or mutation at the appropriate codon. In particular, the truncated NS1 proteins have from 1-60 amino acids, 1-70 amino acids, 1-80 amino acids, 1-90 amino acids (the N-terminal amino acid is 1), and preferably 90 amino acids; from 1- 100 amino acids, and
10 preferably 99 amino acids; from 1-110 amino acids; from 1-120 amino acids; or from 1-130 amino acids, and preferably 124 amino acids of the wildtype NS1 gene product.

The present invention also relates to any genetically engineered influenza virus in which the NS1 gene product has
15 been modified by truncation or modification of the NS1 protein that confers upon the mutant viruses the following phenotypes: the ability of the viruses to grow to high titers in unconventional substrates, such as 6-7 day old chicken eggs, or the ability of the viruses to induce a host
20 interferon response. For influenza A viruses, these include, but are not limited to: viruses having an NS1 truncations.

The present invention includes the use of naturally occurring mutant influenza viruses A or B having the attenuated phenotype, as well as influenza virus strains
25 engineered to contain such mutations responsible for the attenuated phenotype. For influenza A viruses, these include, but are not limited to: viruses having an NS1 of 124 amino acids (Norton et al., 1987, Virology 156:204-213, which is incorporated by reference herein in its entirety).
30 For influenza B viruses, these include, but are not limited to: viruses having an NS1 truncation mutant comprising 127 amino acids derived from the N-terminus (B/201) (Norton et al., 1987, Virology 156:204-213, which is incorporated by reference herein in its entirety), and viruses having an NS1
35 truncation mutant comprising 90 amino acids derived from the N-terminus (B/AWBY-234) (Tobita et al., 1990, Virology 174:314-19, which is incorporated by reference herein in its entirety). The present invention encompasses the use of naturally occurring mutants analogous to NS1/38, NS1/80, NS1/124, (Egorov, et al., 1998, J. Virol. 72(8):6437-41) as

well as the naturally occurring mutants, A/Turkey/ORE/71, B/201 or B/AWBY-234.

The attenuated influenza virus may be further engineered to express antigens of other vaccine strains (e.g., using reverse genetics or reassortment). Alternatively, the attenuated influenza viruses may be engineered, using reverse genetics or reassortment with genetically engineered viruses, to express completely foreign epitopes, e.g., antigens of other infectious pathogens, tumor antigens, or targeting antigens. Since the NS RNA segment is the shortest among the eight viral RNAs, it is possible that the NS RNA will tolerate longer insertions of heterologous sequences than other viral genes. Moreover, the NS RNA segment directs the synthesis of high levels of protein in infected cells, suggesting that it would be an ideal segment for insertions of foreign antigens. However, in accordance with the present invention, any one of the eight segments of influenza viruses may be used for the insertion of heterologous sequences. For example, where surface antigen presentation is desired, segments encoding structural proteins, e.g., HA or NA may be used.

5.2 HOST-RESTRICTION BASED SELECTION SYSTEM

The invention encompasses methods of selecting viruses which have the desired phenotype, i.e., viruses which have low or no IFN antagonist activity, whether obtained from natural variants, spontaneous variants (i.e., variants which evolve during virus propagation), mutagenized natural variants, reassortants and/or genetically engineered viruses. Such viruses can be best screened in differential growth assays that compare growth in IFN-deficient versus IFN-competent host systems. Viruses which demonstrate better growth in the IFN-deficient systems versus IFN competent systems are selected; preferably, viruses which grow to titers at least one log greater in IFN-deficient systems as compared to an IFN-competent system are selected.

Alternatively, the viruses can be screened using IFN assay systems e.g., transcription based assay systems in which reporter gene expression is controlled by an IFN-

responsive promoter. Reporter gene expression in infected versus uninfected cells can be measured to identify viruses which efficiently induce an IFN response, but which are unable to antagonize the IFN response. In a preferred
5 embodiment, however, differential growth assays are used to select viruses having the desired phenotype, since the host system used (IFN-competent versus IFN-deficient) applies the appropriate selection pressure.

For example, growth of virus (as measured by titer) can
10 be compared in a variety of cells, cell lines, or animal model systems that express IFN and the components of the IFN response, versus cells, cell lines, or animal model systems deficient for IFN or components of the IFN response. To this end, growth of virus in cell lines as VERO cells (which are
15 IFN deficient) versus MDCK cells (which are IFN-competent) can be compared. Alternatively, IFN-deficient cell lines can be derived and established from animals bred or genetically engineered to be deficient in the IFN system (e.g., STAT1 -/- mutant mice). Growth of virus in such cell lines, as
20 compared to IFN-competent cells derived, for example, from wild-type animals (e.g., wild-type mice) can be measured. In yet another embodiment, cell lines which are IFN-competent and known to support the growth of wild type virus can be engineered to be IFN-deficient, (e.g., by knocking out STAT1,
25 IRF3, PKR, etc.) Techniques which are well known in the art for the propagation of viruses in cell lines can be used (see, for example, the working examples infra). Growth of virus in the standard IFN competent cell line versus the IFN deficient genetically engineered cell line can be compared.

30 Animal systems can also be used. For example, for influenza, growth in young, IFN-deficient embryonated eggs, e.g., about 6 to about 8 days old, can be compared to growth in older, IFN-competent eggs, e.g. about 10 to 12 days old. To this end, techniques well known in the art for infection
35 and propagation in eggs can be used (e.g., see working examples, infra). Alternatively, growth in IFN-deficient STAT1 -/- mice can be compared to IFN-competent wild type mice. In yet another alternative, growth in IFN-deficient embryonated eggs produced by, for example, STAT1 -/-

transgenic fowl can be compared to growth in IFN-competent eggs produced by wild-type fowl.

For purposes of screening, however, transient IFN-deficient systems can be used in lieu of genetically manipulated systems. For example, the host system can be treated with compounds that inhibit IFN production and/or components of the IFN response (e.g., drugs, antibodies against IFN, antibodies against IFN-receptor, inhibitors of PKR, antisense molecules and ribozymes, etc.). Growth of virus can be compared in IFN-competent untreated controls versus IFN-deficient treated systems. For example, older eggs which are IFN-competent can be pretreated with such drugs prior to infection with the virus to be screened. Growth is compared to that achieved in untreated control eggs of the same age.

The screening methods of the invention provide a simple and easy screen to identify mutant viruses with abolished IFN antagonist activity by the inability of the mutant virus to grow in IFN-responsive environments, as compared to the ability of the mutant virus to grow in IFN-deficient environments. The screening methods of the invention may also be used to identify mutant viruses with altered, but not abolished IFN antagonist activity by measuring the ability of the mutant virus to grow in both IFN-responsive e.g., 10-day old embryonated eggs or MDCK cells and IFN-deficient environments e.g., 6-to-7-day old embryonated eggs or Vero cells. For example, influenza viruses showing at least one log lower titers in 10-days-old eggs versus 6-7 days old eggs will be considered impaired in their ability to inhibit the IFN response. In another example, influenza viruses showing at least one log lower titer in 12 day old eggs (which mount a high IFN response) versus 10 day old eggs (which mount a moderate IFN response) are considered partially impaired in their ability to antagonize the IFN response, and are considered attractive vaccine candidates.

The selection methods of the invention also encompass identifying those mutant viruses which induce IFN responses. In accordance with the selection methods of the invention, induction of IFN responses may be measured by assaying levels of IFN expression or expression of target genes or reporter

genes induced by IFN following infection with the mutant virus or activation of transactivators involved in the IFN expression and/or the IFN response.

In yet another embodiment of the selection systems of the invention, induction of IFN responses may be determined by measuring the phosphorylated state of components of the IFN pathway following infection with the test mutant virus, e.g., IRF-3, which is phosphorylated in response to double-stranded RNA. In response to type I IFN, Jak1 kinase and TyK2 kinase, subunits of the IFN receptor, STAT1, and STAT2 are rapidly tyrosine phosphorylated. Thus, in order to determine whether the mutant virus induces IFN responses, cells, such as 293 cells, are infected with the test mutant virus and following infection, the cells are lysed. IFN pathway components, such as Jak1 kinase or TyK2 kinase, are immunoprecipitated from the infected cell lysates, using specific polyclonal sera or antibodies, and the tyrosine phosphorylated state of the kinase is determined by immunoblot assays with an anti-phosphotyrosine antibody (e.g., see Krishnan et al. 1997, Eur. J. Biochem. 247: 298-305). An enhanced phosphorylated state of any of the components of the IFN pathway following infection with the mutant virus would indicate induction of IFN responses by the mutant virus.

In yet another embodiment, the selection systems of the invention encompass measuring the ability to bind specific DNA sequences or the translocation of transcription factors induced in response to viral infection, e.g., IRF3, STAT1, STAT2, etc. In particular, STAT1 and STAT2 are phosphorylated and translocated from the cytoplasm to the nucleus in response to type I IFN. The ability to bind specific DNA sequences or the translocation of transcription factors can be measured by techniques known to those of skill in the art, e.g., electromobility gel shift assays, cell staining, etc.

In yet another embodiment of the selection systems of the invention, induction of IFN responses may be determined by measuring IFN-dependent transcriptional activation following infection with the test mutant virus. In this embodiment, the expression of genes known to be induced by IFN, e.g., Mx, PKR, 2-5- oligoadenylatesynthetase, major

histocompatibility complex (MHC) class I, etc., can be analyzed by techniques known to those of skill in the art (e.g., northern blots, western blots, PCR, etc.).

Alternatively, test cells such as human embryonic kidney
5 cells or human osteogenic sarcoma cells, are engineered to transiently or constitutively express reporter genes such as luciferase reporter gene or chloramphenicol transferase (CAT) reporter gene under the control of an interferon stimulated response element, such as the IFN-stimulated promoter of the
10 ISG-54K gene (Bluyssen et al., 1994, Eur. J. Biochem. 220:395-402). Cells are infected with the test mutant virus and the level of expression of the reporter gene compared to that in uninfected cells or cells infected with wild-type virus. An increase in the level of expression of the
15 reporter gene following infection with the test virus would indicate that the test mutant virus is inducing an IFN response.

In yet another embodiment, the selection systems of the invention encompass measuring IFN induction by determining
20 whether an extract from the cell or egg infected with the test mutant virus is capable of conferring protective activity against viral infection. More specifically, groups of 10-day old embryonated chicken eggs are infected with the test mutant virus or the wild-type virus. Approximately 15
25 to 20 hours post infection, the allantoic fluid is harvested and tested for IFN activity by determining the highest dilution with protective activity against VSV infection in tissue culture cells, such as CEF cells.

30 5.3 **PROPAGATION OF VIRUS IN INTERFERON DEFICIENT GROWTH SUBSTRATES**

The present invention relates to novel methods and substrates for the propagation of viruses. The invention relates to IFN-deficient substrates and methods for
35 propagating viruses in these substrates. In particular, the invention relates to methods of propagating viruses in immature embryonated eggs, e.g., up to, but less than ten-day-old eggs, preferably six to nine day old eggs, which are normally not used for growing viruses due to their fragile condition and smaller allantoic cavity. In accordance with

the present invention, immature embryonated eggs encompass eggs which as a course of nature are up to, but less than ten-day-old eggs, preferably six-to nine-day-old eggs.

Immature embryonated eggs of the present invention also
5 encompass eggs which artificially mimic immature eggs up to, but less than ten-day-old, as a result of alterations to the growth conditions, e.g., changes in incubation temperatures; treating with drugs; or any other alteration which results in an egg with a retarded development, such that the IFN system
10 is not fully developed as compared with ten-to twelve-day-old eggs. The invention relates to methods of growing any virus in these unconventional growth substrates, including, but not limited to, negative strand RNA viruses, including segmented and non segmented viruses, such as paramyxoviruses (e.g.,
15 Newcastle disease virus, PIV) rhabdoviruses, rabies, orthomyxoviruses (e.g., influenza A and B), bunyaviruses, arenaviruses; positive strand RNA viruses, (e.g., retroviruses); DNA viruses, (e.g., poxviruses)

In accordance with the methods of the present invention,
20 the viruses which may be grown in immature embryonated eggs are selected from naturally occurring strains, variants or mutants, mutagenized virus, reassortants and/or genetically engineered viruses. The methods of the present invention encompass growing the viruses in up to ten-day-old eggs, six
25 to nine-day old eggs, preferably using appropriate growth conditions, (see e.g., growth conditions set forth in Section 6 below) and collecting the progeny virus. The methods of the invention encompassing growth of viruses in immature embryonated eggs, preferably six to nine-day-old eggs, are
30 particularly attractive for growing viruses suitable for use in vaccine formulations.

The invention also encompasses methods and IFN-deficient substrates for the growth and isolation of naturally occurring or engineered mutant viruses having altered IFN
35 antagonist activity. IFN-deficient substrates which can be used to support the growth of the attenuated mutant viruses include, but are not limited to naturally occurring cells, cell lines, animals or embryonated eggs that are IFN deficient, e.g., Vero cells, young embryonated eggs; recombinant cells or cell lines that are engineered to be IFN

deficient, e.g., IFN-deficient cell lines derived from STAT1 knockout mice or other similarly engineered transgenic animals; embryonated eggs obtained from IFN deficient birds, especially fowl (e.g., chickens, ducks, turkeys) including
5 flock that are bred to be IFN-deficient or transgenic birds (e.g., STAT1 knockouts). Alternatively, the host system, cells, cell lines, eggs or animals can be genetically engineered to express transgenes encoding inhibitors of the IFN system, e.g., dominant-negative mutants, such as STAT1
10 lacking the DNA binding domain, antisense RNA, ribozymes, inhibitors of IFN production, inhibitors of IFN signaling, and/or inhibitors of antiviral genes induced by IFN. It should be recognized that animals that are bred or genetically engineered to be IFN deficient will be somewhat
15 immunocompromised, and should be maintained in a controlled, disease free environment. Thus, appropriate measures (including the use of dietary antibiotics) should be taken to limit the risk of exposure to infectious agents of transgenic IFN deficient animals, such as flocks of breeding hens,
20 ducks, turkeys etc. Alternatively, the host system, e.g., cells, cell lines, eggs or animals can be treated with a compound which inhibits IFN production and/or the IFN pathway e.g., drugs, antibodies, antisense molecules, ribozyme molecules targeting the STAT1 gene, and/or antiviral genes
25 induced by IFN.

In accordance with the present invention, immature embryonated chicken eggs encompass eggs which as a course of nature are up to but not yet ten-day-old eggs, preferably six-to nine-day-old eggs; and eggs which artificially mimic
30 immature eggs up to, but not yet ten-day-old, as a result of alterations to the growth conditions, e.g., changes in incubation temperatures; treating with drugs; or any other alteration which results in an egg with a retarded development, such that the IFN system of the egg is not fully
35 developed as compared to 10- to 12-day-old eggs.

5.3.1 NATURAL IFN DEFICIENT SUBSTRATES

In one embodiment, the present invention relates to growing naturally occurring and engineered mutant viruses in unconventional substrates, such as immature embryonated eggs

which have not yet developed an IFN system. Immature embryonated eggs are normally not used to grow virus due to their fragile condition and smaller allantoic volume. The present invention encompasses growing mutant viruses in
5 embryonated eggs less than 10 days old; preferably growing mutated virus in 8-day old embryonated eggs and most preferably, in 6 to 8-day old eggs.

The present invention also encompasses methods of growing and isolating mutated viruses having altered IFN
10 antagonist activity in cells and cell lines which naturally do not have an IFN pathway or have a deficient IFN pathway or have a deficiency in the IFN system e.g., low levels of IFN expression as compared to wild-type cells. In a particular preferred embodiment, the present invention relates to
15 methods of growing mutated viruses having an altered IFN antagonist activity in Vero cells.

5.3.2 GENETICALLY ENGINEERED IFN DEFICIENT SUBSTRATES

20 The present invention relates to methods of growing and isolating mutated viruses having altered IFN antagonist activity in a genetically engineered IFN deficient substrate. The present invention encompasses transgenic avians in which a gene essential to the IFN system is mutated, e.g., STAT1,
25 which would lay eggs that are IFN deficient. The present invention further encompasses avian transgenics which express dominant-negative transcription factors, e.g., STAT1 lacking the DNA binding domain, ribozymes, antisense RNA, inhibitors of IFN production, inhibitors of IFN signaling, and
30 inhibitors of antiviral genes induced in response to IFN. The benefit of using eggs from an IFN-deficient transgenic avian is that the conventional 10 day age eggs may be used to grow the virus which are more stable and have a larger volume due to their larger size. In yet another embodiment, cell
35 lines may be genetically engineered to be IFN deficient. The present invention encompasses cell lines in which a gene essential to the IFN synthesis, IFN pathway, and/or an antiviral gene(s) induced by IFN are mutated, e.g., STAT1.

The invention provides recombinant cell lines or animals, in particular avians, in which one or more genes essential for the IFN pathway, e.g. interferon receptor, STAT1 etc. has been disrupted, i.e., is a "knockout"; the
5 recombinant animal can be any animal but in a preferred embodiment is an avian, e.g. chicken, turkey, hen, duck, etc. (see, e.g., Sang, 1994, Trends Biotechnol. 12:415; Perry, et al., 1993, Transgenic Res. 2:125; Stern, C.D., 1996, Curr Top Microbiol Immunol 212:195-206; and Shuman, 1991, Experientia
10 47:897 for reviews regarding the production of avian transgenics each of which is incorporated by reference herein in its entirety). Such a cell line or animal can be generated by any method known in the art for disrupting a gene on the chromosome of the cell or animal. Such
15 techniques include, but are not limited to pronuclear microinjection (Hoppe & Wagner, 1989, U.S. Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten et al., 1985, Proc. Natl. Acad. Sci., USA 82:6148-6152); gene targeting in embryonic stem cells
20 (Thompson et al., 1989, Cell 56:313); electroporation of embryos (Lo, 1983, Mol Cell. Biol. 3:1803); and sperm-mediated gene transfer (Lavitrano et al., 1989, Cell 57:717); etc. For a review of such techniques, see Gordon, 1989, Transgenic Animals, Intl. Rev. Cytol. 115:171, which is
25 incorporated by reference herein in its entirety.

In particular, a STAT1 knockout animal can be produced by promoting homologous recombination between a STAT1 gene in its chromosome and an exogenous STAT1 gene that has been rendered biologically inactive (preferably by insertion of a
30 heterologous sequence, e.g., an antibiotic resistance gene). Homologous recombination methods for disrupting genes in the mouse genome are described, for example, in Capecchi (1989, Science 244:1288) and Mansour et al. (1988, Nature 336:348-352).

35 Briefly, all or a portion of a STAT1 genomic clone is isolated from genomic DNA from the same species as the knock-out cell or animal. The STAT1 genomic clone can be isolated by any method known in the art for isolation of genomic clones (e.g. by probing a genomic library with a probe derived from a STAT1 sequence such as those sequences

provided in see Meraz et al. 1996, Cell 84: 431-442; Durbin et al. 1996, Cell:84 443-450, and references cited therein). Once the genomic clone is isolated, all or a portion of the clone is introduced into a recombinant vector. Preferably, the portion of the clone introduced into the vector contains at least a portion of an exon of the STAT1 gene, i.e., contains a STAT1 protein coding sequence. A sequence not homologous to the STAT1 sequence, preferably a positive selectable marker, such as a gene encoding an antibiotic resistance gene, is then introduced into the STAT1 gene exon. The selectable marker is preferably operably linked to a promoter, more preferably a constitutive promoter. The non-homologous sequence is introduced anywhere in the STAT1 coding sequence that will disrupt STAT1 activity, e.g., at a position where point mutations or other mutations have been demonstrated to inactivate STAT1 protein function. For example, but not by way of limitation, the non-homologous sequence can be inserted into the coding sequence for the portion of the STAT1 protein containing all or a portion of the kinase domain (e.g., the nucleotide sequence coding for at least 50, 100, 150, 200 or 250 amino acids of the kinase domain).

The positive selectable marker is preferably a neomycin resistance gene (*neo* gene) or a hygromycin resistance gene (*hygro* gene). The promoter may be any promoter known in the art; by way of example the promoter may be the phosphoglycerate kinase (PGK) promoter (Adra et al., 1987, Gene 60:65-74), the *PolIII* promoter (Soriano et al., 1991, Cell 64:693-701), or the MC1 promoter, which is a synthetic promoter designed for expression in embryo-derived stem cells (Thomas & Capecchi, 1987, Cell 51:503-512). Use of a selectable marker, such as an antibiotic resistance gene, allows for the selection of cells that have incorporated the targeting vector (for example, the expression of the *neo* gene product confers resistance to G418, and expression of the *hygro* gene product confers resistance to hygromycin).

In a preferred embodiment, a negative selectable marker for a counterselection step for homologous, as opposed to non-homologous, recombination of the vector is inserted

outside of the STAT1 genomic clone insert. For example, such a negative selectable marker is the HSV thymidine kinase gene (HSV-tk), the expression of which makes cells sensitive to ganciclovir. The negative selectable marker is preferably
5 under the control of a promoter such as, but not limited to the PGK promoter, the PolIII promoter or the MCl promoter.

When homologous recombination occurs, the portions of the vector that are homologous to the STAT1 gene, as well as the non-homologous insert within the STAT1 gene sequences,
10 are incorporated into the STAT1 gene in the chromosome, and the remainder of the vector is lost. Thus, since the negative selectable marker is outside the region of homology with the STAT1 gene, cells in which homologous recombination has occurred (or their progeny), will not contain the
15 negative selectable marker. For example, if the negative selectable marker is the HSV-tk gene, the cells in which homologous recombination has occurred will not express thymidine kinase and will survive exposure to ganciclovir. This procedure permits the selection of cells in which
20 homologous recombination has occurred, as compared to non-homologous recombination in which it is likely that the negative selectable marker is also incorporated into the genome along with the STAT1 sequences and the positive selectable marker. Thus, cells in which non-homologous
25 recombination has occurred would most likely express thymidine kinase and be sensitive to ganciclovir.

Once the targeting vector is prepared, it is linearized with a restriction enzyme for which there is a unique site in the targeting vector, and the linearized vector is introduced
30 into embryo-derived stem (ES) cells (Gossler et al., 1986, Proc. Natl. Acad. Sci. USA 83:9065-9069) by any method known in the art, for example by electroporation. If the targeting vector includes a positive selectable marker and a negative, counterselectable marker, the ES cells in which homologous
35 recombination has occurred can be selected by incubation in selective media. For example, if the selectable markers are the neo resistance gene and the HSV-tk gene, the cells are exposed to G418 (e.g., approximately 300 µg/ml) and ganciclovir (e.g., approximately 2 µM).

Any technique known in the art for genotyping, for example but not limited to Southern blot analysis or the polymerase chain reaction, can be used to confirm that the disrupted STAT1 sequences have homologously recombined into the STAT1 gene in the genome of the ES cells. Because the restriction map of the STAT1 genomic clone is known and the sequence of the STAT1 coding sequence is known (see Meraz et al. 1996, Cell 84:431, Durbin et al. 1996, Cell 84:443-450, all references cited therein), the size of a particular restriction fragment or a PCR amplification product generated from DNA from both the disrupted and non-disrupted alleles can be determined. Thus, by assaying for a restriction fragment or PCR product, the size of which differs between the disrupted and non-disrupted STAT1 gene, one can determine whether homologous recombination has occurred to disrupt the STAT1 gene.

The ES cells with the disrupted STAT1 locus can then be introduced into blastocysts by microinjection and then the blastocysts can be implanted into the uteri of pseudopregnant mice using routine techniques. The animal that develop from the implanted blastocysts are chimeric for the disrupted allele. The chimeric males can be crossed to females, and this cross can be designed such that germline transmission of the allele is linked to transmission of a certain coat color. The germline transmission of the allele can be confirmed by Southern blotting or PCR analysis, as described above, of genomic DNA isolated from tissue samples.

5.3.3 TRANSIENT IFN DEFICIENT SUBSTRATES

The cells, cell lines, animals or eggs can be pre-treated with compounds that inhibit the IFN system. In accordance with the present invention, compounds which inhibit synthesis of IFN, or the activity or the expression of the components of the IFN system can be used to pretreat hosts, e.g., compounds that inhibit the synthesis of IFN, the activity of IFN, the IFN receptor, other targets in the IFN signal transduction pathway, or that inhibit the activity of antiviral genes induced by IFN. Examples of compounds which may be used in accordance with the present invention, include, but are not limited to, nucleic acid molecules,

antibodies, peptides, antagonists of the IFN receptor, inhibitors of the STAT1 pathway, inhibitors of PKR, etc. In accordance with the present invention, nucleic acid molecules include antisense molecules, ribozymes and triple helix
5 molecules that target genes encoding essential components of the IFN system, e.g., STAT1. Nucleic acid molecules also encompass nucleotides encoding dominant negative mutants of components of the IFN system; e.g. prior to infection with the viral mutant, the cells can be transfected with a DNA
10 encoding a truncated, signalling incompetent mutant of the IFN receptor.

Dominant-negative mutants which may be used in accordance with the present invention to inhibit the IFN pathway include kinase deficient versions of Jak1, TyK2 or
15 transcription factors lacking DNA binding domains STAT1, and STAT2 (see, e.g., Krishnan et al., 1997, Eur. J. Biochem. 247: 298-305)

5.4 VACCINE FORMULATIONS

20 The invention encompasses vaccine formulations comprising the attenuated negative strand RNA viruses having an impaired ability to antagonize the cellular IFN response, and a suitable excipient. The virus used in the vaccine formulation may be selected from naturally occurring mutants
25 or variants, mutagenized viruses or genetically engineered viruses. Attenuated strains of segmented RNA viruses can also be generated via reassortment techniques, or by using a combination of the reverse genetics approach and reassortment techniques. Naturally occurring variants include viruses
30 isolated from nature as well as spontaneous occurring variants generated during virus propagation, having an impaired ability to antagonize the cellular IFN response. The attenuated virus can itself be used as the active ingredient in the vaccine formulation. Alternatively, the
35 attenuated virus can be used as the vector or "backbone" of recombinantly produced vaccines. To this end, recombinant techniques such as reverse genetics (or, for segmented viruses, combinations of the reverse genetics and reassortment techniques) may be used to engineer mutations or introduce foreign antigens into the attenuated virus used in

the vaccine formulation. In this way, vaccines can be designed for immunization against strain variants, or in the alternative, against completely different infectious agents or disease antigens.

5 Virtually any heterologous gene sequence may be constructed into the viruses of the invention for use in vaccines. Preferably, epitopes that induce a protective immune response to any of a variety of pathogens, or antigens that bind neutralizing antibodies may be expressed by or as
10 part of the viruses. For example, heterologous gene sequences that can be constructed into the viruses of the invention for use in vaccines include but are not limited to epitopes of human immunodeficiency virus (HIV) such as gp120; hepatitis B virus surface antigen (HBsAg); the glycoproteins
15 of herpes virus (e.g. gD, gE); VP1 of poliovirus; antigenic determinants of non-viral pathogens such as bacteria and parasites, to name but a few. In another embodiment, all or portions of immunoglobulin genes may be expressed. For example, variable regions of anti-idiotypic immunoglobulins
20 that mimic such epitopes may be constructed into the viruses of the invention. In yet another embodiment, tumor associated antigens may be expressed.

 Either a live recombinant viral vaccine or an inactivated recombinant viral vaccine can be formulated. A
25 live vaccine may be preferred because multiplication in the host leads to a prolonged stimulus of similar kind and magnitude to that occurring in natural infections, and therefore, confers substantial, long-lasting immunity. Production of such live recombinant virus vaccine
30 formulations may be accomplished using conventional methods involving propagation of the virus in cell culture or in the allantois of the chick embryo followed by purification.

 Vaccine formulations may include genetically engineered negative strand RNA viruses that have mutations in the NS1 or
35 analogous gene including but not limited to the truncated NS1 influenza mutants described in the working examples, infra. They may also be formulated using natural variants, such as the A/turkey/Ore/71 natural variant of influenza A, or B/201, and B/AWBY-234, which are natural variants of influenza B.

When formulated as a live virus vaccine, a range of about 10^4 pfu to about 5×10^6 pfu per dose should be used.

Many methods may be used to introduce the vaccine formulations described above, these include but are not limited to intranasal, intratracheal, oral, intradermal, intramuscular, intraperitoneal, intravenous, and subcutaneous routes. It may be preferable to introduce the virus vaccine formulation via the natural route of infection of the pathogen for which the vaccine is designed, or via the natural route of infection of the parental attenuated virus. Where a live influenza virus vaccine preparation is used, it may be preferable to introduce the formulation via the natural route of infection for influenza virus. The ability of influenza virus to induce a vigorous secretory and cellular immune response can be used advantageously. For example, infection of the respiratory tract by influenza viruses may induce a strong secretory immune response, for example in the urogenital system, with concomitant protection against a particular disease causing agent.

A vaccine of the present invention, comprising 10^4 - 5×10^6 pfu of mutant viruses with altered IFN antagonist activity, could be administered once. Alternatively, a vaccine of the present invention, comprising 10^4 - 5×10^6 pfu of mutant viruses with altered IFN antagonist activity, could be administered twice or three times with an interval of 2 to 6 months between doses. Alternatively, a vaccine of the present invention, comprising 10^4 - 5×10^6 pfu of mutant viruses with altered IFN antagonist activity, could be administered as often as needed to an animal, preferably a mammal, and more preferably a human being.

5.5 PHARMACEUTICAL COMPOSITIONS

The present invention encompasses pharmaceutical compositions comprising mutant viruses with altered IFN antagonist activity to be used as anti-viral agents or anti-tumor agents or as agents against IFN-sensitive diseases. The pharmaceutical compositions have utility as an anti-viral prophylactic and may be administered to an individual at risk of getting infected or is expected to be exposed to a virus. For example, in the event that a child comes home from school

where he is exposed to several classmates with the flu, a parent would administer the anti-viral pharmaceutical composition of the invention to herself, the child and other family members to prevent viral infection and subsequent illness. People traveling to parts of the world where a certain infectious disease is prevalent (e.g. hepatitis A virus, malaria, etc.) can also be treated.

Alternatively, the pharmaceutical compositions may be used to treat tumors or prevent tumor formation, e.g., in patients who have cancer or in those who are at high risk for developing neoplasms or cancer. For example, patients with cancer can be treated to prevent further tumorigenesis. Alternatively, subjects who are or are expected to be exposed to carcinogens can be treated; individuals involved in environmental cleanups who may be exposed to pollutants (e.g. asbestos) may be treated. Alternatively, individuals who are to be exposed to radiation can be treated prior to exposure and thereafter (e.g. patients exposed to high dose radiation or who must take carcinogenic drugs).

The use of the attenuated viruses of the invention as antitumor agents is based on the Applicants' discovery that an attenuated influenza virus mutant containing a deletion in its IFN-antagonist gene is able to reduce tumor formation in mice. The antitumor properties of the invention can be at least partially related to their ability to induce IFN and IFN responses. Alternatively, the antitumor properties of the attenuated viruses of the invention can be related to their ability to specifically grow in and kill tumor cells, many of which are known to have deficiencies in the IFN system. Regardless of the molecular mechanism(s) responsible for the antitumor properties, the attenuated viruses of the invention might be used to treat tumors or to prevent tumor formation.

The present invention further encompasses the mutant viruses with an altered IFN-antagonist phenotype which are targeted to specific organs, tissues and/or cells in the body in order to induce therapeutic or prophylactic effects locally. One advantage of such an approach is that the IFN-inducing viruses of the invention are targeted to specific sites, e.g. the location of a tumor, to induce IFN in a site

specific manner for a therapeutic effect rather than inducing IFN systemically which may have toxic effects.

The mutant IFN-inducing viruses of the invention may be engineered using the methods described herein to express
5 proteins or peptides which would target the viruses to a particular site. In a preferred embodiment, the IFN-inducing viruses would be targeted to sites of tumors. In such an embodiment, the mutant viruses can be engineered to express the antigen combining site of an antibody which recognized
10 the tumor specific antigen, thus targeting the IFN-inducing virus to the tumor. In yet another embodiment, where the tumor to be targeted expresses a hormone receptor, such as breast or ovarian tumors which express estrogen receptors, the IFN-inducing virus may be engineered to express the
15 appropriate hormone. In yet another embodiment, where the tumor to be targeted expresses a receptor to a growth factor, e.g. VEGF, EGF, or PDGF, the IFN-inducing virus may be engineered to express the appropriate growth factor or portion(s) thereof. Thus, in accordance with the invention,
20 the IFN-inducing viruses may be engineered to express any target gene product, including peptides, proteins, such as enzymes, hormones, growth factors, antigens or antibodies, which will function to target the virus to a site in need of anti-viral, antibacterial, anti-microbial or anti-cancer
25 activity.

Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The pharmaceutical compositions of the present invention may be
30 administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be
35 systemic or local. In addition, in a preferred embodiment it may be desirable to introduce the pharmaceutical compositions of the invention into the lungs by any suitable route. Pulmonary administration can also be employed, e.g., by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

In a specific embodiment, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local
5 infusion during surgery, topical application, e.g., in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such
10 as sialastic membranes, or fibers. In one embodiment, administration can be by direct injection at the site (or former site) of a malignant tumor or neoplastic or pre-neoplastic tissue.

In yet another embodiment, the pharmaceutical
15 composition can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, *supra*; Sefton, 1987, CRC Crit. Ref. Biomed. Eng. 14:201; Buchwald et al., 1980, Surgery 88:507; Saudek et al., 1989, N. Engl. J. Med. 321:574). In another embodiment, polymeric materials
20 can be used (see Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger & Peppas, 1983, J. Macromol. Sci. Rev. 25 Macromol. Chem. 23:61; see also Levy et al., 1985, Science 228:190; During et al., 1989, Ann. Neurol. 25:351 (1989); Howard et al., 1989, J. Neurosurg. 71:105). In yet another embodiment, a controlled release system can be placed in proximity of the composition's target, i.e., the lung, thus
30 requiring only a fraction of the systemic dose (see, e.g., Goodson, 1984, in Medical Applications of Controlled Release, *supra*, vol. 2, pp. 115-138). Other controlled release systems are discussed in the review by Langer (1990, Science 249:1527-1533).

35 The pharmaceutical compositions of the present invention comprise a therapeutically effective amount of the attenuated virus, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a

state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeiae for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the pharmaceutical composition is administered. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the Therapeutic, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

The amount of the pharmaceutical composition of the invention which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. However, suitable dosage ranges for administration are generally about 10^4 - 5×10^6 pfu and can be administered once, or multiple times with intervals as often as needed. Pharmaceutical compositions of the present

invention comprising 10^4 - 5×10^6 pfu of mutant viruses with altered IFN antagonist activity, can be administered intranasally, intratracheally, intramuscularly or subcutaneously. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

6. **EXAMPLE: GENERATION AND CHARACTERIZATION OF NS1 TRUNCATION MUTANTS OF INFLUENZA A VIRUS**

10 6.1 **MATERIALS AND METHODS**

Influenza A/PR/8/34 (PR8) virus was propagated in 10-day-old embryonated chicken eggs at 37°C. Influenza A virus 25A-1, a reassortant virus containing the NS segment from the cold-adapted strain A/Leningrad/134/47/57 and the remaining genes from PR8 virus (Egorov et al., 1994, Vopr. Virusol. 39:201-205; Shaw et al., 1996, in Options of the control of influenza III, eds. Brown, Hampson Webster (Elsevier Science) pp. 433-436) was grown in Vero cells at 34°C. The 25A-1 virus is temperature sensitive in mammalian cells, and was used as a helper virus for the rescue of the NS1/99 transfectant virus. Vero cells and MDCK cells maintained in minimal essential medium (MEM) containing 1 µg/ml of trypsin (Difco Laboratories, Detroit, Michigan) were used for influenza virus growth. Vero cells were also used for selection, plaque purification and titration of the NS1/99 virus. MDCK cells were maintained in DMEM (Dulbecco's minimal essential medium) containing 10% heat-inactivated fetal calf serum. Vero cells were grown in AIM-V medium (Life Technologies, Grand Island, NY).

30 The plasmid pT3NS1/99, which contains a 99 amino acid C-terminal truncated form of NS1 was made as follows. First, pPUC19-T3/NS PR8, containing the complete NS gene of PR8 virus flanked by the T3 RNA polymerase promoter and *Bpu*AI restriction site was amplified by reverse PCR (Ochman et al., 1988, Genetics 120:621-623) using the appropriate primers. The obtained cDNA thus containing the truncated NS1 gene was phosphorylated, Klenow treated, self-ligated and propagated in *E. coli* strain TG1. The construct obtained after purification was named pT3NS1/99 and verified by sequencing.

Plasmids for expression of NP, PB1, PB2, and PA proteins of PR8 virus (pHMG-NP, pHMG-PB1, pHMG-PB2, and pHMG-PA) were previously described (Pleschka et al., 1996, J. Virol. 70:4188-4192). pPOLI-NS-RB was made by substituting the CAT
5 open reading frame of pPOLI-CAT-RT (Pleschka et al., 1996, J. Virol. 70:4188-4192) within RT-PCR product derived from the coding region of the NS gene of influenza A/WSN/33 (WSN)virus. This plasmid expresses the NS-specific viral RNA segment of WSN virus under the control of a truncated human
10 polymerase I promoter.

Generation of NS1/99 virus was performed by ribonucleoprotein (RNP) transfection (Luytjes et al., 1989, Cell 59:1107-1113). The RNPs were formed by T3 RNA polymerase transcription from pT3NS1/99 linearized with *BpuAI*
15 in the presence of purified nucleoprotein and polymerase of influenza 25A-1 virus (Enami, et al., 1991, J. Virol. 65:2711-2713). RNP complexes were transfected into Vero cells which were previously infected with 25A-1 virus. Transfected cells were incubated for 18 hours at 37°C, and
20 the supernatant was passaged twice in Vero cells at 40°C and plaque purified three times in Vero cells covered with agar overlay media at 37°C. The isolated NS1/99 virus was analyzed by RT-PCR using specific primers. The wild-type transfectant virus was generated as follows: Vero cells in
25 35-mm dishes were transfected with plasmids pHMG-NP, pHMG-PB1, pHMG-PB2, pHMG-PA and pPOLI-NS-RB, as previously described (Pleschka et al., 1996, J. Virol. 70:4188-4192). Two days post-transfection, cells were infected with 5×10^4 pfu of delNS1 virus and incubated two more days at 37°C.
30 Cell supernatant was passaged once in MDCK cells and twice in chicken embryonated eggs. Transfectant viruses were cloned by limiting dilution in eggs. Genomic RNA from purified NS1/99 transfectant virus was analyzed by polyacrylamide gel electrophoresis, as previously described (Zheng et al., 1996,
35 Virology 217:242-251). Expression of a truncated NS1 protein by NS1/99 virus was verified by immunoprecipitating labeled infected cell extracts using a rabbit polyclonal antisera against NS1.

The allantoic cavity of embryonated chicken eggs, aged 6, 10, and 14 days were inoculated with approximate 10^3 pfu

of PR8, NS1/99, or delNS1 (in which the entire NS1 gene is deleted) viruses, incubated at 37°C for two days, and the viruses present in the allantoic fluid were titrated by hemagglutination (HA) assay.

5 Groups of 5 BALB/c mice (Taconic Farms) were inoculated intranasally with 5×10^6 pfu, 1.5×10^5 pfu, or 5×10^3 pfu of wild-type A/PR/8/34 (PR8) or NS1/99 virus. Inoculations were performed under anesthesia using 50 μ l of MEM containing the appropriate number of plaque forming units of the
10 appropriate virus. Animals were monitored daily, and sacrificed when observed in extremis. In a subsequent experiment, all surviving mice were challenged four weeks later with a dose of 100LD₅₀ of wild-type PR8 virus. All procedures were in accord with NIH guidelines on care and
15 use of laboratory animals.

6.2 RESULTS: ATTENUATION OF INFLUENZA A VIRUSES BY NS1 DELETIONS

Applicants have previously shown that an influenza A
20 virus in which the NS1 gene was deleted (delNS1 virus) is able to grow to titers of approximately 10^7 pfu/ml in cells deficient in type I Interferon (IFN) production, such as Vero cells. However, this virus was impaired in its ability to replicate and cause disease in mice (García-Sastre et al.,
25 1998, Virology 252:324). By contrast, delNS1 virus was able to grow in and kill STAT1 -/- mice. These results demonstrated that the NS1 protein of influenza A virus is a virulence factor involved in the inhibition of the host antiviral responses mediated by type I IFN. The following
30 experiments were conducted to determine whether one could generate influenza viruses with virulence characteristics intermediate between wild-type and delNS1 viruses by deleting portions of the NS1 gene and whether some of these viruses might have optimal characteristics for being used as live
35 attenuated vaccines against influenza viruses, i.e., stability and an appropriate balance between attenuation, immunogenicity and growth in substrates suitable for vaccine preparation, such as embryonated chicken eggs.

In order to test this hypothesis, an influenza A/PR/8/34 (PR8) virus was generated in which the NS1 gene has been

modified in order to direct the expression of a truncated NS1 protein containing only 99 amino acids at the amino terminal in common with the 230 amino acids of the wild-type NS1 protein. This virus (NS1-99) was obtained by RNP

5 transfection of an artificially engineered NS gene using 25A-1 helper virus, as previously described (García-Sastre et al., 1998, Virology 252:324). Analysis of NS1 expression in virus infected cells revealed the truncated nature of the NS1 protein of the NS1-99 virus.

10 The ability of delNS1, NS1-99 and wild-type PR8 viruses to grow in embryonated chicken eggs of different ages was analyzed. The rationale for this experiment comes from the fact that the ability of embryonated eggs to synthesize and to respond to type I IFN under an appropriate stimulus is age
15 dependent. In fact, both IFN inducibility and responsiveness start at an age of approximately 10 days, and then exponentially increase with the age (Sekellick et al., 1990, In Vitro Cell. Dev. Biol. 26:997; Sekellick & Marcus, 1985, J. Interferon Res. 5:657). Thus, the use of eggs of
20 different ages represents a unique system to test the ability of different viruses to inhibit IFN responses. Eggs of 6, 10, and 14 days of age were inoculated with approximately 10^3 pfu of PR8, NS1-99 or delNS1 viruses, incubated at 37°C for 2 days, and the viruses present in the allantoic fluid were
25 titrated by hemagglutination (HA) assay. As shown in Table 3, whereas wild-type virus grew to similar HA titers in embryonated eggs of 6, 10 and 14 days of age, delNS1 only replicated to a detectable HA titer in 6-day-old eggs. By contrast, NS1-99 virus showed an intermediate behavior
30 between delNS1 and wild-type viruses, and was able to grow to HA titers similar to those of wild-type virus in 10-day-old eggs, but not in 14-day-old eggs.

Table 3: Virus replication in embryonated chicken eggs.

5	<u>Virus</u>	Age of eggs:	<u>Hemagglutination titer¹</u>		
			<u>6 days</u>	<u>10 days</u>	<u>14 days</u>
	WT PR8 ²		2,048	4,096	1,071
	NS1/99		N.D. ³	2,048	<2
10	delNS1		64	<2	<2

¹ Titers represent the highest dilution with hemagglutinating activity.

² Wild-type influenza A/PR/8/34 virus.

³ Not determined.

The attenuation characteristics of NS1-99 virus were next determined in mice. For this purpose, groups of 5 BALB/c mice were intranasally infected with 5×10^6 pfu, 1.5×10^5 or 1.5×10^3 pfu of wild-type PR8 or NS1-99 virus. Mice were then monitored during 3 weeks for survival. The results are given in Table 4. NS1-99 virus had an LD50 at least three logs higher than that of wild-type virus.

Table 4. Attenuation of NS1-99 virus in mice

25	<u>Virus</u>	<u>Infecting dose (pfu):</u>	<u>Survivors</u>		
			5×10^6	1.5×10^5	5×10^3
	WT PR8 ¹		1/5	1/5	1/5
	NS1-99		3/5	5/5	5/5

30 ¹ Wild-type Influenza Virus A/PR/8/34.

7. EXAMPLE: GENERATION AND CHARACTERIZATION OF NS1 TRUNCATION MUTANTS IN INFLUENZA B VIRUS

35

7.1 MATERIALS AND METHODS

Experimental details are similar to those in Section 6.1. Two mutant influenza B viruses, B/610B5B/201 (B/201) and B/AWBY-234, 127-amino-acid and 90 amino acids in length (C-terminal truncated NS1 proteins), respectively

(Norton et al., 1987 Virology 156: 204; Tobita et al., 1990 Virology 174:314) were derived from coinfection experiments in tissue culture involving B/Yamagata/1/73 (B/Yam) and A/Aichi/2/68 viruses in the presence of anti-A (H3N2) virus antibody. The growth of the mutant influenza viruses in embryonated eggs of various ages were compared to that of parental virus B/Yam, which possess a wild-type 281-amino-acid NS1 protein. Eggs of 6, 10 and 14 days of age were inoculated with approximately 10^3 pfu of B/Yam, B/201 or B/AW/BY-234 viruses, incubated at 35°C for 2 days, and the viruses present in the allantoic fluid were titrated by an HA assay.

Further, the attenuation characteristics of B/201 and B/AWBY-234 viruses were determined in mice. Groups of three BALB/c mice were intranasally infected with 3×10^5 pfu of wild-type B/Yam, B/201 or B/AWBY-234 mutant viruses, and the ability of these viruses to replicate was determined by measuring viral titers in lungs at day 3 postinfection since wild-type B/Yam does not induce apparent signs of disease in mice.

7.2 RESULTS

Table 5: Influenza B virus replication in embryonated chicken eggs.

<u>Virus</u>	<u>Age of eggs:</u>	<u>Hemagglutination titer</u>		
		<u>6 days</u>	<u>10 days</u>	<u>14 days</u>
B/Yam		362	256	<2
B/201		32	<2	<2
B/AWBY-234		8	<2	<2

The results from the growth of the mutant and wild-type influenza B viruses in embryonated chicken eggs, shown in Table 5, demonstrate that, as in the case with influenza A viruses, a carboxy-terminal truncation of the NS1 of influenza B virus is responsible for a lower replication yield in older embryonated chicken eggs which mount an

efficient IFN response. This finding indicates that the NS1 of influenza B virus is also involved in inhibiting the IFN responses of the host, and that deletions on the NS1 gene of influenza B virus result in an attenuated phenotype.

5 The results from the replication experiments in mice are given in Table 6. B/201 and B/AWBY-234 virus titers were approximately three logs of magnitude lower than B/Yam titers, indicating that truncations of the carboxy-terminal domain of the NS1 of influenza B virus are responsible for an
10 attenuated phenotype in mice.

Table 6. Influenza B virus replication in mouse lungs

15	<u>Virus</u>	<u>Lung titers at day 3 postinfection (pfu/lung)</u>		
	B/Yam	2×10^4	1×10^4	3×10^4
	B/201	30	<10	60
	B/AWBY-234	<10	40	<10

20

**8. PROTECTION AGAINST WILD-TYPE INFLUENZA
VIRUS INFECTION IN MICE IMMUNIZED WITH
INFLUENZA A AND B VIRUSES CONTAINING
DELETIONS IN THEIR NS1 PROTEINS**

In order to determine whether mice immunized with
25 attenuated influenza A and B viruses containing truncated NS1 proteins were protected against challenge with their respective wild-type viruses the following experiment was carried out. BALB/c mice were immunized intranasally with
A/NS1-99 virus and three weeks later they were infected with
30 100 LD₅₀ of wild-type influenza A/PR/8/34 virus. Immunized animals were protected against death, while all control naive mice died after the challenge (see Table 7). In a second
experiment, BALB/c mice were intranasally immunized with the
influenza B viruses B/201 or B/AWBY-234, expressing truncated
35 NS1 proteins. Three weeks later the mice were challenged with 3×10^5 pfu wild-type influenza B/Yam/1/73 virus. Since this strain of influenza B virus does not induce disease symptoms in mice, the degree of protection was determined by measuring virus titers in lungs at day 3 post-challenge.

While naive control animals had titers around 10^4 pfu/lung, viruses were not detected in lungs of immunized animals (see Table 8). These findings suggest that influenza A as well as influenza B viruses containing modified NS1 genes are able to induce an immune response in mice which is fully protective against subsequent wild-type virus challenge.

Table 7. Survival of mice immunized with influenza A/NS1-99 virus after challenge with 100 LD₅₀ of wild-type influenza A/PR/8/34 virus.

<u>Immunizing Dose of A/NS1-99 Virus</u>	<u>Number of Survivors/Total</u>
5×10^6 pfu	3/3
1.5×10^5 pfu	4/4
PBS	0/5

Table 8. Lung titers in mice immunized with influenza B/201 and B/AWBY-234 viruses after challenge with 3×10^5 pfu of wild-type influenza B/Yamagata/73 virus.

<u>Immunizing Dose</u>	<u>Lung titers (pfu/lung)</u>
3×10^5 pfu of B/201	$<10^1, <10^1, <10^1, <10^1, <10^1$
3×10^5 pfu of B/AWBY-234	$<10^1, <10^1, <10^1, <10^1, <10^1$
PBS	$2.5 \times 10^4, 1 \times 10^4, 1.7 \times 10^4, 3 \times 10^4, 5 \times 10^4$

9. EXAMPLE: INDUCTION OF TYPE I INTERFERON IN EMBRYONATED EGGS INFECTED WITH DELNS1 VIRUS

The ability of delNS1 virus, an influenza A virus lacking the NS1 gene, to induce type I IFN secretion in embryonated chicken eggs was next determined. For this purpose, groups of two 10-days-old embryonated chicken eggs were infected with 5×10^3 pfu of delNS1 or wild-type PR8 viruses. Eighteen hours postincubation at 37°C, the allantoic fluid was harvested and dialyzed against acid pH overnight, to inactivate infectious viruses. After acid pH treatment, samples were dialyzed against PBS, and they were tested for IFN activity by determining the highest dilution with protective activity against VSV infection (approximately

200 pfu) in CEF cells. The results shown in Table 9 indicate that in the absence of NS1, influenza A viruses are higher inducers of IFN.

Table 9. Induction of IFN in eggs.

5	<u>Virus</u>	<u>IFN (U/ml)</u>
	PR8	<16, <16
	delNS1	400, 400
	mock	<16, <16

10

10. EXAMPLE: ANTIVIRAL ACTIVITY OF delNS1 VIRUS

Elimination of the IFN antagonist (NS1) gene from influenza A virus may result in a virus with the ability to induce high levels of IFN. If this is the case, delNS1 virus will "interfere" with the replication of IFN-sensitive viruses. In order to test this possibility, Applicants investigated the ability of delNS1 virus to inhibit the replication of influenza A/WSN/33 (WSN) virus, a commonly used laboratory strain of influenza virus, in eggs. As can be seen in Figure 1, treatment with only 2 pfu of delNS1 virus was able to reduce the final titers of WSN virus in the allantoic fluid by one log. In addition, treatment with 2×10^4 pfu of delNS1 virus resulted in practically complete abrogation of WSN replication in eggs. DelNS1 virus was also able to interfere with the replication in eggs of other influenza A virus strains (H1N1 and H3N2), influenza B virus and a different virus such as Sendai virus (Figure 2).

Encouraged by these results, Applicants next determined the ability of delNS1 virus to interfere with wild-type influenza virus replication in mice. Although type I IFN treatment in tissue culture prevents influenza A virus replication *in vitro*, treatment of mice with IFN is not able to inhibit the replication of influenza viruses (Haller, 1981, Current Top Microbiol Immunol 92:25-52). This is true for most inbred strains of mice, except for A2G mice. A2G mice, as well as a significant proportion of wild mice (approximately 75%), contain at least one intact Mx1 allele, while most laboratory strains are Mx1 -/- (Haller, 1986,

Current Top Microbiol Immunol 127:331-337). The Mx1 protein, which is a homologue of the human MxA protein (Aebi, 1989, Mol. Cell. Biol. 11:5062), is a potent inhibitor of influenza virus replication (Haller, 1980, Nature 283:660). This protein is not constitutively expressed, but its expression is transcriptionally induced by type I IFN. Thus, A2G mice can be used to test the ability of IFN-inducers to stimulate an antiviral response against influenza A viruses (Haller, 1981, Current Top Microbiol Immunol 92:25-52).

10 Applicants intranasally infected eight 4-week-old A2G mice with 5×10^6 pfu of a highly pathogenic influenza A/PR/8/34 virus isolate (Haller, 1981, Current Top Microbiol Immunol 92:25-52). Half of the mice received an intranasal treatment with 5×10^6 pfu of delNS1 at -24h with respect to the PR8

15 infection. The other four mice were treated with PBS. Body weight changes and survival was monitored. These results demonstrate that delNS1 treatment was able to protect A2G mice against influenza virus-induced death and body weight lost. The same treatment was not effective in Mx1 -/- mice

20 indicating that the mechanism of viral protection was Mx1, i.e. IFN, mediated.

11. EXAMPLE: ANTITUMOR PROPERTIES OF DELNS1 VIRUS IN MICE

25 Given that type I IFN and/or inducers of type I IFN have been shown to have antitumor activities (Belardelli and Gresser, 1996 Immunology Today 17: 369-372; Qin et al., 1998, Proc. Natl. Acad. Sci. 95: 14411-14416), it is possible that treatment of tumors with delNS1 virus might mediate tumor

30 regression. Alternatively, delNS1 virus might have oncolytic properties, i.e., it may be able to specifically grow in and kill tumor cells, many of which are known to have deficiencies in the IFN system. In order to test anti-tumor activity of delNS1 virus, the following experiment was

35 conducted using murine carcinoma cell line CT26.WT in a mouse tumor model for pulmonary metastasis (Restifo et al., 1998 Virology 249:89-97). 5×10^5 CT26.WT cells were injected intravenously into twelve 6-weeks-old BALB/c mice. Half of the mice were treated intranasally with 10^6 pfu of delNS1 virus every 24 hours at days 1, 2 and 3 postinoculation.

Twelve days after tumor injection, mice were sacrificed and lung metastases were enumerated. As shown in Table 10, delNS1 treatment mediated a significant regression of murine pulmonary metastases.

5

Table 10. Antitumor activity of delNS1 virus in BALB/C mice injected with CT26.WT tumor cells.

		<u>Number of pulmonary metastases</u>	
		<u>PBS-treated</u>	<u>delNS1-treated</u>
10	Mouse 1	>250	120
	Mouse 2	>250	28
	Mouse 3	>250	9
	Mouse 4	>250	6
15	Mouse 5	>250	2
	Mouse 6	>250	1

12. **EXAMPLE: THE NS1 PROTEIN INHIBITS THE TRANSLOCATION OF IRF-3 DURING INFLUENZA VIRUS INFECTION**

The results described herein suggest that the NS1 protein of influenza virus is responsible for the inhibition of the type I IFN response against the virus, and that mutations/deletions in this protein result in attenuated viruses due to an enhanced IFN response during infection. It is known that synthesis of type I IFN during viral infection can be triggered by double-stranded RNA (dsRNA). IRF-3 is a transcription factor which is usually found in an inactive form in the cytoplasm of mammalian cells. Double-stranded RNA induces the phosphorylation (activation) of the transcription factor IRF-3, resulting in its translocation to the nucleus, where it induces transcription of specific genes, including genes coding for type I IFN (Weaver et al., 1998, Mol. Cell. Biol. 18:1359). In order to determine if NS1 of influenza is acting on IRF-3, IRF-3 localization in CV1 cells infected with wild-type PR8 or with delNS1 influenza A virus was monitored. Figure 3 shows that IRF-3 translocation is minimal in PR8-infected cells (in fewer than 10% of the cells). In contrast, approximately 90% of delNS1-

infected cells showed nuclear localization of IRF-3. Strikingly, it was possible to partially inhibit the IRF-3 translocation in delNS1-infected cells by expressing NS1 from a plasmid in trans. The results demonstrate that the NS1 of influenza A virus is able to inhibit IRF-3 translocation in virus-infected cells. It is likely that the NS1 of influenza virus prevents dsRNA-mediated activation of IRF-3 by sequestering the dsRNA generated during viral infection, thus resulting in an inhibition of IFN synthesis.

10 The present invention is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and any constructs or viruses which are functionally equivalent are within the scope of this invention. Indeed, 15 various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

20 Various references are cited herein, the disclosures of which are incorporated by reference in their entireties.

25

30

35

What is claimed:

- 5 1. A method for propagating viruses comprising:
 - (a) growing the virus in immature embryonated eggs; and
 - (b) collecting progeny virus,with the proviso that said virus is not influenza C.
10
2. The method of Claim 1 wherein the eggs are less than ten-day-old embryonated chicken eggs.
- 15 3. The method of Claim 2 wherein the eggs are six to nine-day-old embryonated chicken eggs.
4. The method of Claim 1 wherein the virus is a negative strand RNA virus.
20
5. The method of Claim 4 wherein the virus is a segmented negative strand RNA virus.
6. The method of Claim 5 wherein the virus is
25 influenza A or B.
7. The method of Claim 4 wherein the virus is a non-segmented negative strand RNA virus.
- 30 8. The method of Claim 7 wherein the virus is NDV, PIV, or VSV.
9. The method of Claim 1 wherein the virus is a DNA virus.
35
10. The method of Claim 9 wherein the virus is a poxvirus.
11. A method for propagating viruses comprising

- (a) growing the virus in an interferon deficient substrate, and
(b) collecting progeny virus,
with the proviso that said virus is not influenza C and the
5 interferon deficient substrate is not Vero cells and is not
STAT1(-) cell lines.

12. The method of Claim 11 wherein the substrate is
embryonated chicken eggs less than ten-days-old.

10

13. The method of Claim 12 wherein the substrate is six
to nine-day old embryonated chicken eggs.

14. The method of Claim 13 wherein the virus is a
15 negative strand RNA virus.

15. The method of Claim 14 wherein the virus is a
segmented negative strand RNA virus.

20 16. The method of Claim 15 wherein the virus is
influenza A or B.

17. The method of Claim 11 wherein the virus is a non-
segmented negative strand RNA virus.

25

18. The method of Claim 17 wherein the virus is NDV,
PIV, or VSV.

19. The method of Claim 11 wherein the virus is a DNA
30 virus.

20. The method of Claim 19 wherein the virus is a
poxvirus.

35 21. The method of Claim 1 or 11 wherein the propagated
virus has an attenuated phenotype.

22. The methods of Claim 1 or 11 wherein the propagated
virus is grown for use in vaccine formulations.

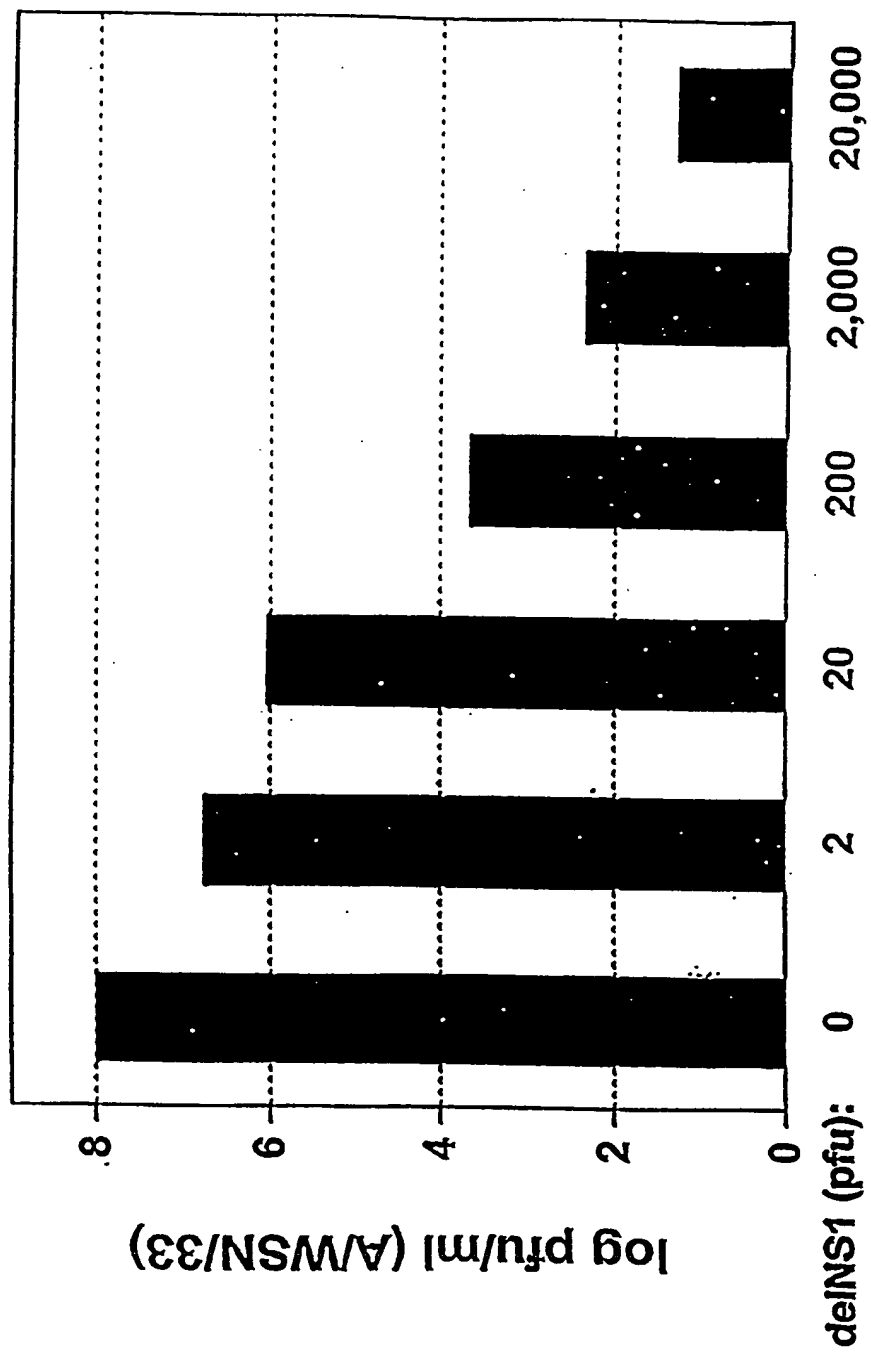


FIG. 1

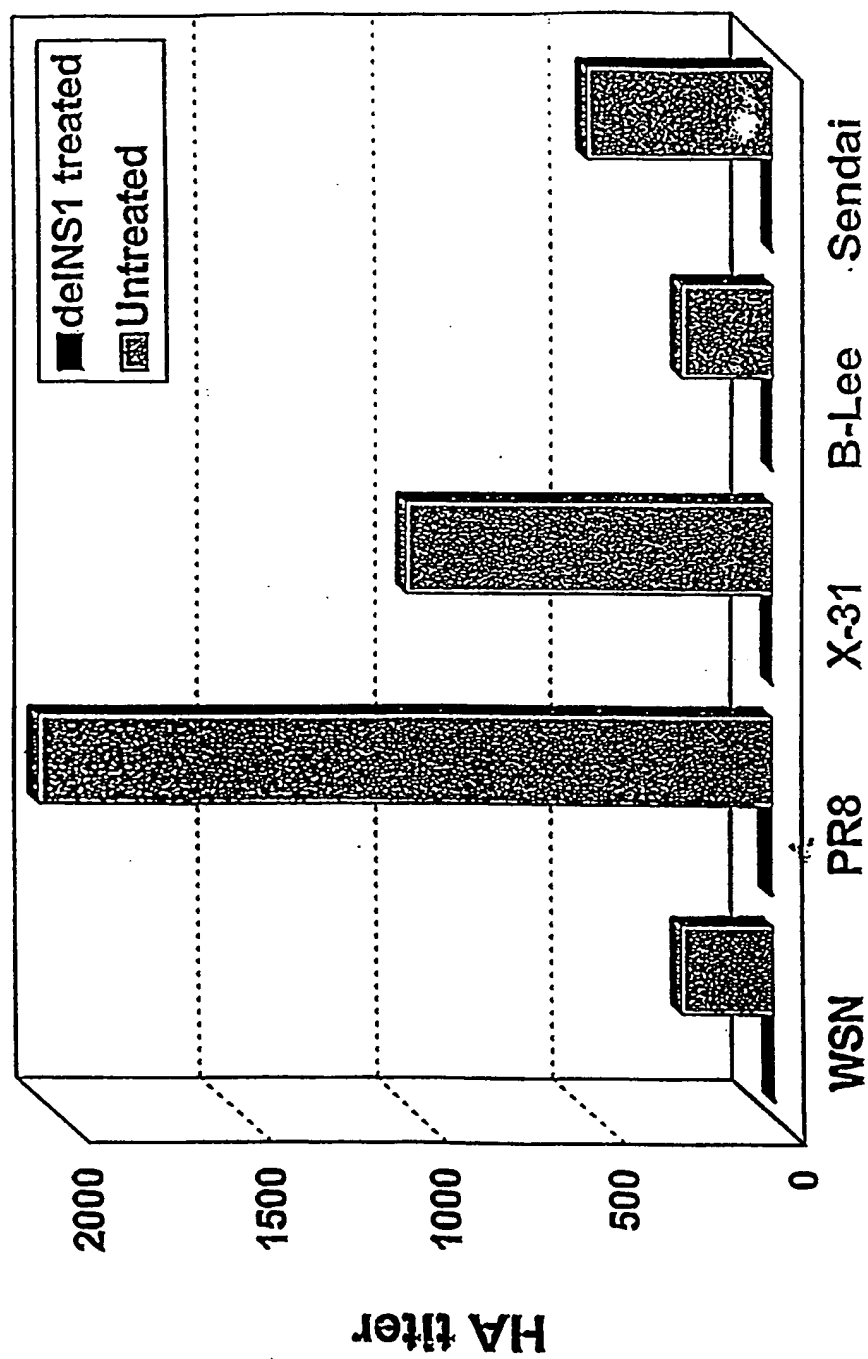


FIG. 2

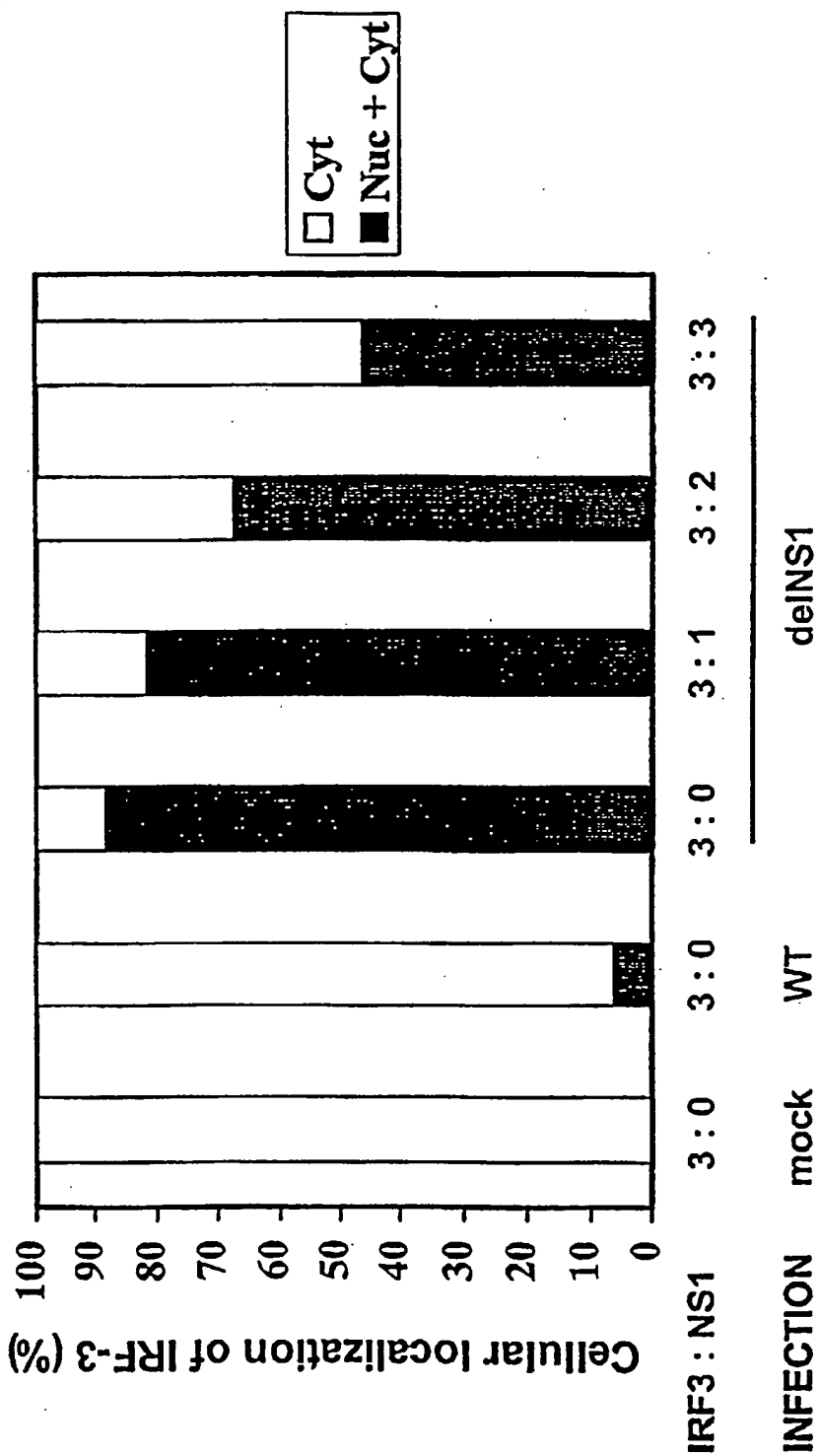


FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/13142

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 7/00; A61K 39/12

US CL : 435/235.1; 424/204.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/235.1; 424/204.1, 209.1, 211.1, 214.1, 232.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4,071,618 A (KONOBE et al) 31 January 1978, col. 8, lines 41-68.	1, 4-7, 11, 21, 22
X	US 4,659,569 A (MITSUHASHI et al) 21 April 1987, col. 6, lines 14-24; col. 8, line 65 - col. 9, line 5.	1-8, 11-14, 17, 18, 21, 22
X	GOODPASTURE et al. The cultivation of vaccine and other viruses in the chorioallantoic membrane of chick embryos. Science. 1934, Vol. 74, No. 1919, pages 371-372, see entire document, particularly page 371, column 2, third paragraph.	1-3, 9-13, 19-22
X	JP 59-39831 A (KK BISEIBUTSU KAGAKU KENKYUSHO) 05 March 1984, page 2, col. 1, lines 6-10.	1-6, 11-16, 22

☐

Further documents are listed in the continuation of Box C.

☐

See patent family annex.

*

Special categories of cited documents:

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later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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document defining the general state of the art which is not considered to be of particular relevance

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A

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Date of the actual completion of the international search

06 AUGUST 1999

Date of mailing of the international search report

21 OCT 1999

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/13142

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, BIOSIS, CAB, DERWENT WEST WORLD PATENTS INDEX. SEARCH TERMS: VIRUS, VIRUSES, EGG, EGGS, 6, 7, 8, 9, SIX, SEVEN, EIGHT, NINE, NOT PY=(1998 OR 1999), DAY, DAYS, CHICK, CHICKS, CHICKEN, EMBRYO, EMBRYOS, INFLUENZA, "A", "B", AFRICAN HORSE, HEN, DNA, RNA, FOWLPOX, FP, FPV, POX7, VACCINIA, MVA

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